Célia Cristina Oliveira Sarmento

BSc in Biochemistry

Natural deep eutectic systems – a new delivery system for ocular drugs

Dissertation for the Master degree in Biochemistry for Health

Supervisor: Ana Rita Jesus, PhD, NOVA School of Science and Technology

Co-supervisor: Ana Rita Duarte, Associate Professor, NOVA School of Science and Technology

October, 2022

Célia Cristina Oliveira Sarmento

BSc in Biochemistry

Natural deep eutectic systems – a new delivery system for ocular drugs

Dissertation for the Master degree in Biochemistry for Health

Supervisor: Ana Rita Jesus, PhD, NOVA School of Science and Technology

Co-supervisor: Ana Rita Duarte, Associate Professor, NOVA School of Science and Technology

Examination Committee:

Chair: Maria Teresa Nunes Mangas Catarino, Auxiliary Professor, NOVA School of Science and Technology
Rapporteur: Joana Marques Marto, Auxiliary Professor, Auxiliary Professor, Faculdade de Farmácia, Universidade de Lisboa
Supervisor: Ana Rita Xavier de Jesus Gameiro, PhD, NOVA School of Science and Technology

NOVA School of Science and Technology

October, 2022

NATURAL DEEP EUTECTIC SYSTEMS – A NEW DELIVERY SYSTEM FOR OCULAR DRUGS

ACKNOWLEDGMENTS

Firstly, and foremost, I would like to express my gratitude to my supervisors Dr. Ana Rita Jesus and Prof. Ana Rita Duarte for the opportunity of working on a project that I simply loved. What an amazing journey. A gigantic thank you for the immense patience, time, help and support throughout this long year.

I would like to thank every single member of the DES.Solve group, from Des.Solve Lab and as well as from the Biocatalysis & Bioenergy Group for the welcoming and the amazing fellowship. You made me feel part of the big family in a way that I've never imagined. To Olga that started this adventure with me.

Thank you to my friend Afonso, with whom laughed and cried through this year, and it is always here after all these years in college. To Patrício and Matias for being who you are, all the moments and support.

An enormous thank you to my grandma that made these 6 years all possible, it is beyond words.

To my great grandma, my "bisa", will always be my star in the sky.

Then, to my parents, that believed in me once and once again, and made the possible in order to get me here. Thank you for your patience and for understanding the long times I have been away from home.

Lastly, for you, Miguel, for everything. This is also yours; this is for you. This journey would not have been possible without your love and support. And as I once said, "In this world there is not enough thanks for you".

ABSTRACT

The major goal of this work was to study the potential of natural deep eutectic systems (NADES) as new media for ocular formulations mainly due to their high viscosity, as it is an important aspect related to drug retention time in ocular formulations. Therefore, different systems composed of combinations of sugars, polyols, amino acids, and choline derivatives were prepared.

NADES were characterized through a series of various techniques to evaluate if they could be used as ocular delivery systems via topical instillation, namely rheological and physicochemical studies were carried out.

In terms of viscosity, it was observed that aqueous solutions of NADES showed viscosity values within the standard values to be used as an ocular drug delivery system, i.e., within the range of 0.8 to 1.2 mPa.s. The pH of the solutions was measured, showing that the values are near the physiological value (pH 7.4). Other parameters were analyzed and when compared with commercial samples our aqueous solutions of NADES have contact angles between 100.7 and 109.9 º when measured in a hydrophobic surface, refractive indexes of 1.3404 to 1.3491, osmolality values ranged from 412 to 1883 $mOsmolkg^{-1}$, and surface tension between 60.28 and 71.57 mN/m. All values were within the values obtained for commercial samples. The cytotoxicity assays demonstrated that, in general, these systems are biocompatible, promoting cell viability, i.e., above 80% metabolic activity when compared to control, after 24h incubation in ARPE-19 cells, and therefore do not cause harm in ocular cells.

Furthermore, rutin, resveratrol and taurine, three relevant antioxidants for ocular applications, were successfully dissolved in NADES, and their stability was determined at predetermined timepoints. The results showed that the antioxidant activity is not significantly altered up to 6 months and using NADES as excipients might be useful to extend the shelf-life of ocular drops. Overall, the results suggest that NADES are potential excipients to be used in ocular formulations.

Keywords: ocular diseases; oxidative stress; NADES; natural antioxidants; eye drops

RESUMO

O principal objetivo deste trabalho foi estudar o potencial dos sistemas eutéticos naturais (NADES), como novos meios para formulações oculares principalmente devido à sua elevada viscosidade, uma vez que é um aspeto importante relacionado com o tempo de retenção de fármacos nas formulações oculares. Assim, foram preparados diferentes sistemas compostos por combinações de açúcares, polióis, aminoácidos, e derivados de colina.

Os NADES foram caraterizados através de várias técnicas a fim de avaliar se poderiam ser utilizados como sistemas de administração ocular através de instilação tópica, nomeadamente estudos reológicos e físico-químicos.

Em termos de viscosidade, observou-se que as soluções aquosas de NADES apresentavam valores de viscosidade dentro dos valores padrão a serem utilizados como sistema de administração de fármacos oculares, isto é, no intervalo de 0,8 a 1,2 mPa.s. O pH das soluções foi medido, mostrando que os valores estão próximos do valor fisiológico (pH 7,4).

Outros parâmetros foram analisados e quando comparados com amostras comerciais, as nossas soluções aquosas de NADES têm ângulos de contacto semelhantes, entre 100,7 e 109,9 º quando adquidos sob uma superfície hidrofóbica, os índices de refração de 1,3404 a 1,3491, os valores de osmolalidade variaram de 412 a 1883 $mOsmolkg^{-1}$, e a tensão superficial entre 60,28 e 71,57 mN/m. De uma forma geral todos se encontravam dentro dos valores obtidos para amostras comerciais. Os ensaios de citotoxicidade demostraram que, em geral, estes sistemas são biocompatíveis, promovendo a viabilidade celular acima de 80% de actividade metabólica quando comparada com o controlo, após incubação 24h em células ARPE-19, e portanto não causam danos nas células oculares.

Além disso, a rutina, o resveratrol e a taurina, três antioxidantes relevantes para aplicações oculares, foram dissolvidos com sucesso nos NADES, e a sua estabilidade foi determinada em momentos pré-determinados ao longo do tempo. Os resultados mostraram que a actividade antioxidante não é significativamente alterada até 6 meses e a utilização de NADES como excipientes pode ser útil para prolongar o prazo de validade das gotas oculares. Resumindo, os resultados sugerem que os NADES são potenciais excipientes a serem utilizados em formulações oculares.

Palavras-chave: doenças oculares; stress oxidativo; NADES; antioxidantes naturais; gotas oftálmicas

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMD	Age-related macular degeneration
API	Active pharmaceutical ingredients
AT	Artificial tears
BEG	Betaine: Ethylene glycol
Bet	Betaine
BTGW	Betaine:Trehalose:Glycerol:Water
BTW	Betaine:Trehalose:Water
DED	Dry eye disease
DES	Deep eutectic systems
DNA	Deoxyribonucleic acid
EG	Ethylene glycol
FBS	Fetal bovine serum
Fru	Fructose
Glc	Glucose
Gly	Glycerol
GSH	Glutathione
HA	Hyaluronic acid
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HPMC	Hydroxypropyl methyl cellulose
NAC	*N*-acetylcysteine
NADES	Natural deep eutectic systems
PBS	Phosphate buffer saline
Pro	Proline
PS	Penicillin-streptomycin
PTFE	Polytetrafluoroethylene
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RSA	Radicals scavenging activity
Rut	Rutin

Rvst	Resveratrol
SD	Standard Deviation
Sorb	Sorbitol
Suc	Sucrose
SOD	Superoxide dismutase
Tau	Taurine
Treh	Trehalose
UV	Ultraviolet light
W	Water

1. INTRODUCTION

1.1 Present time and eye diseases

With the increasing life expectancy and consecutive growth in the average population's age, the necessities and needs of people are shifting to different conditions, mainly in the direction of non-communicable diseases where ophthalmic conditions are included. [1], [2] Blindness and visual impairment substantially influence people's quality of life, and they are also important in terms of social and public health. [3] They are usually linked to less economic, educational, and career prospects and an increased chance of mortality. [1], [2] In older people, visual impairment also exacerbates comorbidities such as cognitive impairment and the risk of falling. [1]

The World Health Organization (WHO) estimates that at least 2.2 billion people worldwide have some kind of vision impairment or blindness and from these, at least 1 billion have a condition that can be prevented and/or treated through early diagnosis. [4] In addition, the Vision Loss Expert Group of the Global Burden of Disease Study estimated that 295 million people have moderate to severe vision impairment and 43 million are classified as blind. [1] Nevertheless, vision impairment affects all ages, but the most affected population group is those aged 50 or more. [2] In Europe, it is estimated that there are 1.3 million blind people, with a further 10 million people living with moderate to severe vision impairment in the same age group. With these numbers, it was also estimated that the cost of productivity loss in the European Union (EU) was around €30 billion for blindness and moderate to severe vision impairment. [3]

The prevalent causes of global blindness in 2020 were cataracts, under-corrected refractive error, followed by glaucoma, age-related macular degeneration (AMD), and diabetic retinopathy. In the elderly, glaucoma and AMD were the most prominent. In moderate to severe vision impairment, under-corrected refractive error was the principal contributor in ages below 70, and cataracts above. Geographically, cataract was the largest cause of blindness in ages above 50 except in high-income countries, located mostly in western Europe and the Asia Pacific such as Japan, Korea, and Australia, where glaucoma has a higher number of cases. [2] The latter aspect can be justified by the fact that some of these disorders are treatable, namely cataracts that can be treated with the right medical care. However, in

areas where this condition is most prevalent, the majority of people lack access to these services.[2], [5]

1.2 Eye

The eye is an organ of the visual system with unique physiology and anatomical structure which results from the complexity of various tissues (Figure 1.1). [6]

Overall, the eye can be divided into anterior and posterior segments. The first includes the cornea, iris, pupil, conjunctiva, ciliary body, an anterior chamber filled with aqueous humor, trabecular meshwork, and lens. The posterior segment comprises the vitreous chamber filled with vitreous humor, sclera, choroid, retina, macula, and optic nerve. [7], [8]

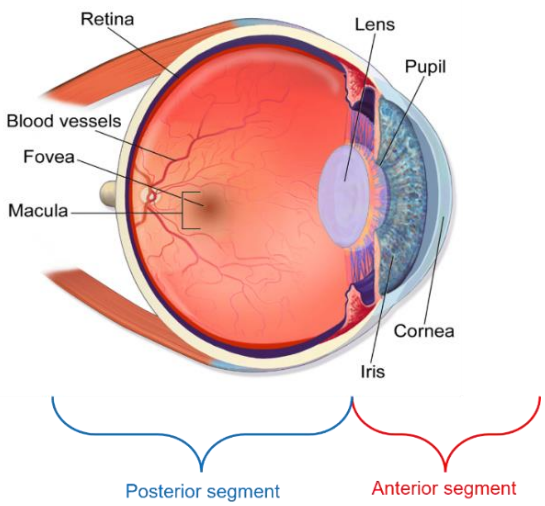

Figure 1.1 - Eye Anatomy (adapted from Blausen.com staff [9]).

At the eye surface, there are tears that lubricate and protect the eye and there are three layers, also denominated as the tear film, i.e., the oily layer on the exterior composed of lipids, the watery layer full of proteins and electrolytes in the middle, and the mucin layer on the inside. When the eye blinks, they work together to maintain the eye moist. [8], [10], [11]

The light is focused into the eye through the cornea, anterior chamber, pupil, and lens. Then, the light hits the retina after passing through the vitreous chamber. The main processes take place in the retina. In this light-sensitive tissue, there is a particular type of cells called photoreceptors that are responsible for phototransduction. There are two types of photoreceptors: rods and cones. The first only perceive black and white and provide night vision because they are particularly sensitive to low-intensity light. Cones are responsible for visual precision and for distinguishing colors, being the most relevant with

central vision. Each of these photoreceptors consists of an outer and an inner segment. In the outer segment are located the photosensitizers, specific protein pigments/chromophores such as rhodopsin, that transform light into chemical signals that are sent as electrical impulses through the optical nerve into the visual cortex, the part of the brain responsible for vision, a process known as phototransduction. [8], [10]

1.3 Eye Diseases

These are several conditions that affect the eye such as cataracts, glaucoma, and dry eye in the anterior segment, and diabetic retinopathy and macular degeneration in the posterior segment. (Figure 1.2) Although there are other eye diseases, in the context of this work, only the diseases that are influenced by oxidative stress resulting from photodegradation and aging, which are also the most common moderate to severe eye diseases, will be discussed.

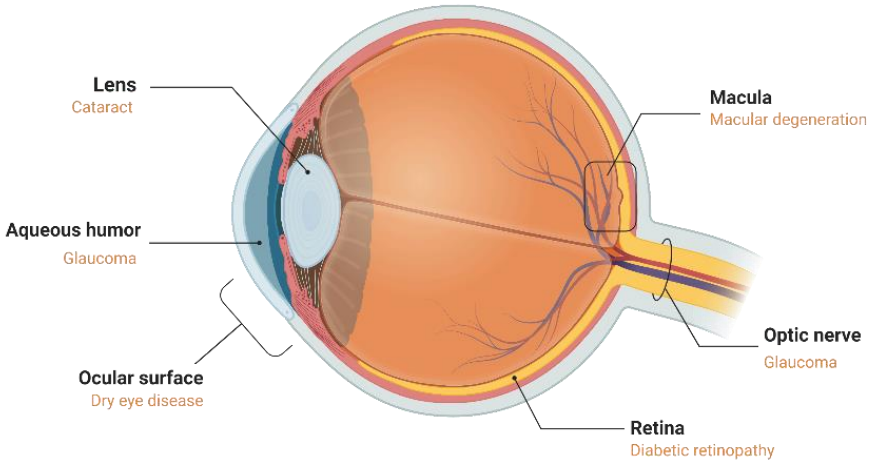

Figure 1.2 - Eye diseases affected by oxidative stress (Created with BioRender.com).

1.3.1 Cataracts

Cataract disease is the opacification of the eye's lens. In normal conditions, the lens transparency is maintained due to the unique protein packing and lens cells architecture. [12] Lens transparency and refractive index are strongly influenced by these structural proteins. [13] A cataract occurs when this architecture collapses. The development of the condition is credited to the deposition of aggregated proteins in the eye lens and plasma membrane disruption in lens fiber cells forming an insoluble turbid protein provoking clouding, light scattering, and obstruction of vision. [12], [14] However, they have more causes from abnormalities, metabolic disorders, osmotic pressure changes, trauma, and drug-induced changes to its main cause and risk: aging. There are other risk factors such as cigarette smoking,

exposure to sunlight, alcohol use, and nutritional supplements. [15] Based on the cause, cataracts can be classified into pediatric cataracts, age-related cataracts, and cataracts secondary to other causes. [6] Based on the location/form of the opaqueness they can be of three main types: nuclear, cortical, and posterior subcapsular cataracts.

Several pathogenic mechanisms can involve Na^+/K^+ adenosine triphosphatase decrease in activity, oxidative stress, lens protein aggregation, advanced glycation end-products, trauma, polyol pathway activation and genetic abnormalities. [16], [17] Age-related cataracts result mainly due to oxidation. They develop from reactive oxygen species (ROS)-induced damage in the lens cells leading to oxidation of proteins, deoxyribonucleic acid (DNA) damage, and lipid peroxidation. The lens relies on redox balance to maintain clarity, and there is substantial evidence that mitochondrial malfunction and ROS imbalance play a role in the genesis of cataracts. [14]

1.3.2 Glaucoma

Glaucoma is a chronic degenerative optic neuropathy. It is characterized by progressive degeneration of the retinal ganglion cells. These cells belong to the central nervous system, whose degeneration results in gradual optical atrophy and vision loss. [18] Glaucoma generally results from an increase in the intraocular pressure of the aqueous humor in the anterior chamber which causes retinal ganglion cells apoptosis. [19] This condition is divided into different types: open-angle glaucoma, angle-closure glaucoma, and normal tension glaucoma. [6], [8] This definition is based on the iridocorneal angle, an end position of the aqueous humor. [20]

Open-angle glaucoma develops when the aqueous humor drains slowly, creating an excess of fluid in the eye and a rise in intraocular pressure. This form is the most frequent and is asymptomatic in the early stages before progressing to progressive visual loss, which is difficult to diagnose. Closed-angle glaucoma occurs when the iris restricts the trabecular meshwork, causing an increase in ocular pressure having its peak when the drainage angle gets blocked, leading to acute attack with severe eye pain, abrupt blurry vision, headache, nausea, and vomiting and consequent loss of vision. [6], [8], [18], [20] The latter type is like the first without high levels of intraocular pressure. These are normally primary diseases, however, there is also secondary glaucoma that results from trauma, inflammation, tumor, specific drugs such as corticosteroids, or other conditions such as pigment dispersion or pseudo-exfoliation. [18] Usually, intraocular pressure is the factor that is controlled and treated in order to access the condition. [6] The major risk factors are age on a large scale, as well as ethnicity (African-Americans), genetic heritability, hypertension, trauma, uveitis, myopia, diabetes, and topical corticosteroids. [20] ROS influence occurs within trabecular meshwork and slows the drainage of the aqueous humor, increasing intraocular pressure. Moreover, oxidative stress also induces changes in the apoptotic pathway in the trabecular meshwork allowing the progression of the disease. Retinal ganglion cells have a high concentration of mitochondria, being the mitochondrial DNA the one damaged due to ROS increasing the causes of these cells' death.[17], [21]

1.3.3 Macular degeneration

Macular degeneration is a progressive condition that affects the RPE, mainly the macula, and the photoreceptor cells. This disease can be distinguished between AMD and Stargardt disease, being the first most prevalent. As implied, AMD occurs mainly due to the aging process, while the second normally urges before the age of 20. [22]

AMD has multiple causes and usually results from the combination of multiple contributions: aging, genetic predisposition, metabolic, environmental (smoking, ultraviolet light (UV), blue light), and function factors, which connect with oxidative stress and accumulation of lipofuscin in RPE. [22]–[24] Furthermore, there are other risk factors such as smoking, high blood pressure, high cholesterol, and obesity. Other characteristics such as light skin color, gender, and light eye color are also risk factors for AMD. [8] Stargardt disease, on the other side, usually results from a genetic predisposition. [22]

The retinal pigment epithelium (RPE) also has a protective function against photooxidation and prevents the entry of toxic compounds into the retina. ROS led to RPE cell death due to damage to its mitochondrial DNA as mentioned before. Being this tissue is highly oxygen demanding and is exposed to irradiation, these cells are vulnerable to oxidative damage being one of the roots associated with the pathological progress of macular degeneration. [25]

1.3.4 Diabetic retinopathy

Diabetic retinopathy is one of the most severe complications of diabetes mellitus and is becoming more common as diabetic patients have longer lifespans. Glucose harms the retina through repeated acute and cumulative alterations. Damage in the retina's microvasculature causes the blood vessels to enlarge and leak fluid, and gradually, if this continues, new vessels begin to form, which eventually causes the retina to detach. [26] This damage is triggered by chronic hyperglycemia combined with other risk factors, namely hypertension, dyslipidemia, and cataract surgery. Dysfunction in the mitochondria is likewise a component important in this cascade, hence oxidative stress and inflammation. [27] When diabetic macular edema and proliferative diabetic retinopathy demonstrate macular ischemia, vitreous hemorrhage, and tractional retinal detachment in diabetic retinopathy patients, may occur visual loss. [20]

1.3.5 Dry eye disease

Dry eye disease (DED) is a frequent condition that affects one out of every three people worldwide. It does affect the tears and the surface of the eye. It is defined as loss of homeostasis of the tear film, which causes pain, visual disruption, and tear film instability. This condition is additionally characterized

by an increase in tear film osmolarity and consequent general and chronic inflammation of the ocular surface. [28]–[30] There are two types of DED characterized by its cause: aqueous deficiency and/or increased evaporation. [30] Likewise the previous conditions, DED incidence increases with age, and it is more common in ages over 50. Because of such incidences, it is sometimes considered age-related, majorly due to the implication that oxidative stress has in the development of this condition. Oxidative stress is therefore linked with damaged ocular tissues and inflammatory pathways. [31] Inflammation leads to reduced tear secretion and corneal neuropathy hence the disruption of the balance of the tear film and thus the dry eye condition. [32] Still, there are more risk factors and many of them are environmental (exposure to pollutants, UV radiation, ozone), but also prolonged use of eye drops such as those used in glaucoma treatment present issues to the tear film. [32], [33]

1.4 Eye drug delivery systems

The eye has such a complex structure that it is still a challenge for drug delivery. [34] Anatomic and physiologic barriers are the main obstacles to the success of ocular drug delivery. [35] The fundamentals of any drug delivery system are to improve drug absorption and minimize dosing frequency while maintaining therapeutic drug concentrations at the target site constant and accurately with the least adverse effects possible. It should also be simple to handle and manufacture, stable throughout the ocular surface and the many dynamic and static barriers, biodegradable and biocompatible, with a long shelf life. [35], [36]

When dealing with anterior segment illnesses the most often used non-invasive mode of medicine delivery is topical application. Ocular formulations such as solutions, suspensions, emulsions, gels, and ointments account for 90% of the marketed ophthalmic formulations. [37], [38] They are relatively non-invasive, simple to use, minimizing systemic adverse effects, and drug dosage since it is administered locally rather than as a systemic medication, thus avoiding first-pass metabolism and being patient-compliant. [34], [35] However, the most significant disadvantage is their limited ocular bioavailability, which accounts for less than 5% of the administered dose reaching deeper eye structures due to many anatomical and physiological constraints such as high tear turnover rate, nasolacrimal drainage, reflex blinking, and reduced absorption owing to the tear-film barrier. [35], [36] (Figure 1.3) As a consequence, it requires frequent administration over a long period of time and therefore, poor patient compliance, thus decreasing treatment efficacy. [29] These factors also make it extremely difficult to formulate topical eye drops for the posterior segment pathologies. [34], [35] To improve drug bioavailability, an ophthalmic formulation requires a higher precorneal residence time and enhanced drug permeation.

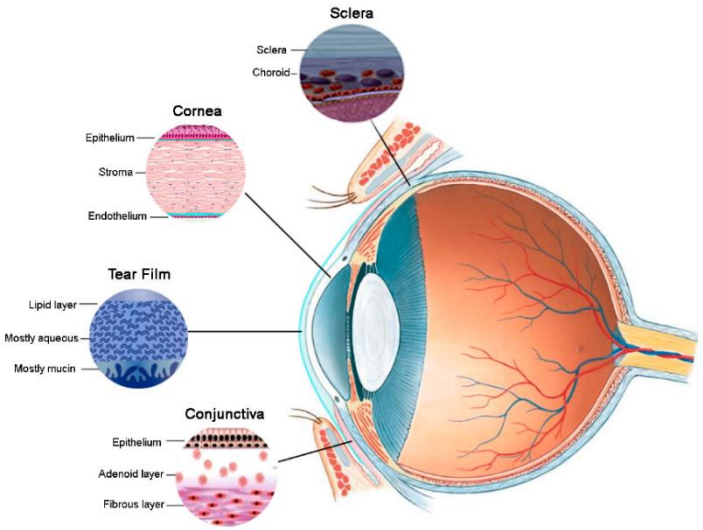

Figure 1.3 - Eye barriers to topical administration of drugs [29].

Targeting posterior segment diseases, usually, intravitreal injections, periocular injections, and systemic administration are prescribed. However, these options present various drawbacks. The most popular and frequently advised method for the administration of medication to treat posterior ocular disorders is the intravitreal injection, however, the necessity for frequent eye punctures has several negative side effects, including endophthalmitis, hemorrhage, retinal detachment, and low patient tolerance. Transscleral drug delivery with periocular administration is an alternative route. Although transscleral distribution is relatively simple, less invasive, and more patient-friendly, ocular static and dynamic obstacles impair drug permeation. Systemic administration becomes inviable due to the tiny volume of the eye in comparison to the rest of the organ and the presence of blood retinal barriers.

To overcome all these barriers, various alterations to the drugs and their formulations were performed throughout the years. There are two main routes in these modifications: drug and formulation properties. In Table 1.1 there is a summary of these drug delivery systems, their advantages, and disadvantages. Relatively to the first, the major example is prodrugs. This technique alters the drug's physicochemical characteristics to improve drug permeation through the cornea, increase drug shelf life, and stabilize the drug chemically and metabolically. The mechanism involves cellular enzymes that break the prodrug into the active drug only at the target site. [35], [36], [39] Another way to overcome the limitations of eye drops is to tune the formulation properties. Hydrophobic drugs are extremely difficult to solubilize and therefore have low bioavailability in the eye. A way to resolve this question is by using cyclodextrins, cyclic oligosaccharides organized in a cone-like form. These compounds allow hydrophobic drugs to form complexes working as carriers in topical aqueous solutions. [36], [40], [41]

Table 1.1 – Common eye drug delivery systems

Drug Delivery System	Advantages	Drawbacks	Commercial Examples	Ref
Prodrugs	- Corneal permeation improved - Bioavailability improved several times - Dose reduced - Able to be used within other delivery systems	- Hard to formulate into aqueous eye drops. - Undesirable pharmacokinetic characteristics - Difficulties in the adjustment of the ocular drug delivery properties	- Dipivefrine (Propine®, Allergan) - Latanoprost (Xalatan®, Pfizer)	[35], [39]
Cyclodextrins	- Prolonged drug release - Bioavailability improved in some cases - Able to be used within other delivery systems - Can reach posterior segment by topical instillation	- Functional only in specific cases - Sometimes decreases bioavailability - Can be toxic to the cornea - Occurs precorneal loss	- Chloramphenicol (Clorocil®, Edol) - Diclofenac (Voltaren Ophthalmic®, Novartis)	[35], [36], [40]
Ocular implants and inserts	- Can be used for topical or systemic therapy - Controlled drug delivery for a prolonged period - Sustained localized drug delivery - Reduces drug application frequency - Less susceptibility to nasolacrimal drainage - Higher drug concentrations in the cornea - Can reduce treatment time - Good stability over time	- Can occur rejection - The feeling of a foreign body in the eye - Sporadic failures in introducing and using inserts - Can occur blurred vision	- Ocusert®, Alza Corporation - Lacrisert®, Merck - Ocufit® SR, Escalon Medical Corp. - Surodex®, Allergan - Vitrasert®, Bausch and Lomb Inc. - Ozurdex®, Allergan	[40]–[42]

Table 1.1 – Common eye drug delivery systems (cont.)

Drug Delivery System	Advantages	Drawbacks	Commercial Examples	Ref
Nanoparticles	- Small size leads to low irritation - Increased corneal permeation - Prolonged release, eliminating frequent drug administration - Bioavailability improved - Used for posterior segment diseases by periocular administration	- Can be toxic - Can lead to tissue accumulation blocking drainage - Size, charge, and surface dependent - Low capacity - Rapid clearance when using ocular and periocular formulations. - Need for intravitreal administration when delivered to the posterior segment - Some instability and difficulties in encapsulating some drugs	- Ocusolin™, AlphaRx (in preclinical trials)	[34], [36], [42]
Liposomes	- Biodegradable and biocompatible - Able to encapsulate both hydrophilic and lipophilic drug moieties - Demonstrated effectiveness for both anterior and posterior segments - Properties can be tuned with lipid composition, size, surface charge, and preparation method - Promote close contact with ocular tissues	- Poor stability and a short half-life - Poor reproducibility - Low drug-entrapment efficiency	- ClaryMist®, Savant - Ocusoft®, Ocusoft - Visudyne®, QLT Ophthalmics	[34], [36], [40], [42]
Drug-eluting contact lens	- Prolonged drug release - Enhance drug penetration across the corneal epithelium vs conventional eye drops - Highest drug bioavailability	- Issues with safety and efficacy - Prolonged use can lead to corneal toxicity - Need improvements in oxygen diffusion, microbial resistance, and effective and continuous drug release - Not commercialized yet	-	[35]

In the additive area, the use of viscosity and permeation enhancers is a common option to improve drugs' precorneal residence time and permeation. This will be further explored in the next section.

Despite existing some other considered "old" technologies, the fact is that all of them have issues and the search for new options continues to be a reality. More recently, it was developed some technologies namely drug-eluting contact lenses, as the name implies, there are contact lenses coated with drugs to apply to the eyeball sustainedly for longer periods. [35], [41] In the same window, ocular inserts appeared, which are solid or semi-solid formulations that are placed in the cornea. They are constituted by polymers, which specify if the implant is soluble, insoluble, and biodegradable and the drug is introduced into the polymer as a solution or a dispersion. These devices must be implanted into the eye via a small surgery. [41], [42] Additionally, as nanotechnology emerged, several nanocarriers in form of colloids and suspensions also appeared as new drug delivery approaches including nanoparticles, nanosuspensions, liposomes, nanomicelles, dendrimers, and others that derived from these. [34], [36] From these only nanoparticles and liposomes are described in this work because are the two most investigated recently. Nanoparticles are, as the name suggests, particles with a diameter of fewer than 1 μm, and in the ophthalmic drug delivery context, made of a variety of biodegradable and biocompatible substances, including natural or synthetic polymers with mucoadhesive properties where is added the drug in solution or trapped. [36], [41] Liposomes are spherical vesicles made up of concentric phospholipid bilayers. As they are composed of lipids, they are non-toxic and biodegradable. Similarly, as cell membranes, these vesicles are amphiphilic and thus, can deliver both hydrophilic and hydrophobic compounds. Depending on their composition, they can present any charge at their surface. In this context of ocular drug delivery, the most relevant are the positively charged liposomes, due to negatively charged mucin at the corneal surface. [36] Several systems result from ideas from these 2 nanotechnologies but still at a small scale.

1.5 Topical eye drops and challenges in formulation

Ocular drops are the most common method used for the treatment of anterior segment disorders as discussed and here it will be explored in more detail as the objective of this thesis is to create an ocular formulation for the prevention and/or treatment of these conditions.

The first challenges were already mentioned in the previous section, namely the precorneal residence time and drug permeation. Commercial eye drops usually have a volume of at least 40 μL, however, as the eye starts to blink and tear to expel foreign substances in order to restore the normal tear volume, which is around 30 μL at its maximum, results in the remaining of less than 10 μL of the drop applied following a single blink, which only has around 5 min to pass the first barrier until it gets full washed out. Additionally, if are administered two or more eye drops, the residence time is even lower, thus they end up competing in the precorneal space. Interestingly, if one single drop contains more than one drug in

equivalent amounts, this competition does not occur and eyedrop formulations can simultaneously treat more than one condition and improve treatment effectiveness. [29]

Normally in topical eye drops many additives are used such as viscosity and permeation enhancers to overcome the rapid nasolacrimal drainage. The first promotes the goal characteristics by increasing the formulations' viscosity. As described before, when drops are applied, the fluid instillation results in an increase in tear volume. The instilled fluid has a viscosity similar to tears which is about 1.5 mPa.s and gets eliminated in minutes. In order to increase that time residence time, is required to prolong the residence time for the instilled fluid. [43] It has been suggested that eye drops' viscosity to maintain precorneal residence in humans is 10 mPa.s. [44] Additionally, it has been reported that only once the fluid viscosity exceeded a crucial value of roughly 10 mPa.s, the retention begin to rise, but the relative increase in retention got lower at extremely high viscosities. [43], [45] Another important aspect is that high viscosities can also cause discomfort and damage in ocular tissue due to the increased shear stress during blinking. [43]

Some examples of viscosity enhancers used in ocular formulations are hydroxy methyl cellulose (HMC), hydroxy ethyl cellulose (HEC), sodium carboxy methyl cellulose (NaCMC), hyaluronic acid (HA), polyalcohol (PA), and hydroxypropyl methyl cellulose (HPMC) in low concentrations, below 1%. [34], [35], [40], [46] The usual concentrations of these polymers are not harmful to vision since their refractive indices are comparable to those of the lacrimal fluid. Besides polymers, there also used Pluronics®, which also improves drug solubility and enhances the viscosity of topical formulations. To improve retention time, it is necessary viscosities of about 20mPa.s. [46]

Permeation enhancers temporarily modify the cornea, improving its absorption and its surface activity. These other additives are preservatives, surface active, chelating agents, and bile salts. Chemicals such as benzalkonium chloride, polyoxyethylene glycol, ethers, and ethylenediamine tetra acetic acid disodium salt (EDTA) are some examples of permeation enhancers. These compounds usually present local toxicity provoking damage in the cornea, being used with limitation, and cannot be used in long term. [35], [41], [46] An alternative to the use of these compounds is by utilizing single-dose units, nevertheless, they are harder to use and have a higher cost. [29]

Another important aspect is tonicity. In ophthalmic formulations, the medicine is dissolved in sterile water to achieve an isotonic solution. This property is only relevant in aqueous solutions. Lacrimal fluid tonicity corresponds to a 0.9% sodium chloride (NaCl) solution. Nevertheless, the eye tolerates a large range of values without discomfort and favors hypertonic solutions. The most frequently used excipients are NaCl, potassium chloride (KCl), sodium nitrate ($NaNO_3$), or potassium nitrate (KNO_3). When dealing with pH, amphiphilic drugs are the ones that have the highest penetration rate. In the cornea, a drug can permeate through the epithelium and there are preferred undissociated and lipophilic molecules, whereas, in the stroma, is preferred dissociated and hydrophilic molecules. [46] Tonicity, the osmotic pressure between two compartments, is directly compared with osmolarity, and osmolarity, is considered "the effective osmolality". The terms "osmolality" and "osmolarity" in the case of dilute solutions with water can be used interchangeably, especially in tears, where the difference is less than

5%. [47] In perfect and ideal conditions, the formulation should have the same pH as lacrimal fluid, 7.4. When applied, a slightly acid solution does not harm, until the lacrimal fluid's natural buffering capacity is exceeded and eye drops are rapidly removed by the body's buffer system. Other aspects are sterility. Preservatives must be present in aqueous ophthalmic treatments delivered in multidose vials in the proper amounts to maintain sterility for a month. Lastly, to prevent corneal irritations, eye solutions must be free from particles, being at least on a micro-scale. [46]

1.6 Eye diseases and oxidative photodegradation and stress

The eye is a structure that is highly metabolically active due to its rich content of mitochondria and the high metabolic rate of the photoreceptors and constant light absorption. Oxidative and photooxidative processes along with oxidative stress are important factors that for many years were not considered by those investigating ocular disease. [17], [48], [49]

Oxidative stress can be defined as an imbalance between prooxidants and antioxidants in favor of the first. [50] Prooxidants are the compounds that promote this condition either by producing free radicals or by inhibiting the antioxidant system. Free radicals are generated by ROS, reactive nitrogen species, and reactive carbonyl species, where ROS are the most relevant. [50] On the other side, antioxidants are compounds that directly scavenge those radicals or work indirectly to up-regulate antioxidant defenses or suppress radical formation. [51] These will be explored in the next section. Oxidative stress usually results from excessive ROS production, mitochondrial dysfunction, impaired antioxidant system, or a combination of the previous factors. [52]

ROS are represented by superoxide anion radical (O2•-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and singlet oxygen ($^1O_2$), which are generated as a byproduct of the respiratory chain in mitochondria, in photochemical and enzymatic reactions. (Figure 1.4) These eventually provoke autophagy, apoptosis, and also necrosis, which consequently causes tissues and organs to become dysfunctional. [52]

In normal conditions, this oxidative damage is minimized with endogenous antioxidants and repair processes. With aging, there is an increase in mitochondrial dysfunction and oxidative damage together with decreases in antioxidant and repair mechanisms. Oxidative damage leads to dysfunction and cell loss, resulting in visual impairment. [27] Ocular diseases such as the ones described previously in section 1.3. are consequences.

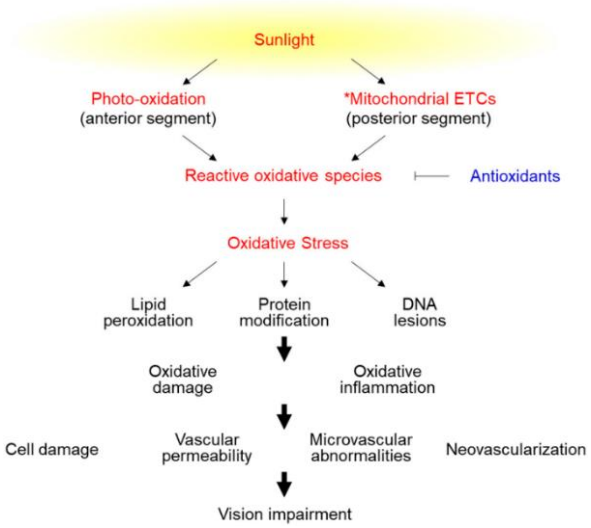

Figure 1.4 - ROS effects in the eye [49] (Mitochondrial ETCs: Mitochondrial electron transport chain).

As visible in Figure 1.4, the anterior segment is where there is more photooxidation, which can be explained by the fact that these are the tissues where occur most of the UV absorption. Also, its superficial location, where the cornea is the most affected, makes them exposed to atmospheric oxygen, thus enduring more persistently ROS and oxidative stress. [52], [53] Additionally, the lens has some of the oldest cells in the body that cannot be replaced, making it especially vulnerable to aging damage, leading to loss of transparency and consecutive visual impairment. [14]

In the posterior segment, as UV does not reach those tissues, ROS and consecutive oxidative stress have different sources. [53] The RPE accumulates more photosensitizers with age. Lipofuscin is a fluorescent mixture of partially digested proteins and lipids that accumulates over time in the lysosomal compartment of the RPE and can act as a photosensitizer, absorbing large quantities of light, which consequently generates ROS, as well as its accumulation, has some other toxic effects that damage not only the RPE but the whole retina. [53], [54] Furthermore, the retina, which is in constant light exposure, is the area of the human body that consumes the most oxygen in the whole body. The inner segments of the photoreceptor cells are densely packed with mitochondria which provide the adenosine triphosphate (ATP) necessary for the ionic pumps that participate in the visual transduction to the brain. [27] With age, various mitochondrial dysfunctions occur, and the mitochondrial respiratory chain function decreases in efficiency. Mitochondrial oxidative stress can also accelerate the release of cytochrome C, an apoptosis precursor, into the cytosol. ROS causes mitochondrial dysfunction and apoptosis, playing a significant role in retinal diseases. [22]

1.7 Antioxidants to prevent and treat ocular diseases

As many diseases are diagnosed in advanced stages, it is necessary for an improvement of diagnosis but also there is a necessity to improve therapeutic approaches and the prevention of these eye illnesses. [17], [55]

The enrolment and importance of oxidative stress in many eye diseases have been discussed throughout this work. As shown in the previous section, the cell's defenses are carried by its antioxidant defense system. This system is composed of antioxidants that directly scavenge ROS, and work indirectly in order to inhibit, delay its formation, or reduce or eliminate the effects of oxidative stress by prevention, inhibition, and repair. [50], [51] Antioxidants can be enzymatic and nonenzymatic. The first are mainly intracellular and includes enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase. [49], [52] Nonenzymatic antioxidants are present both in cells and in extracellular fluids and can be proteins or small molecules namely ascorbic acid (vitamin C), α-tocopherol (vitamin E), and glutathione (GSH). All are extremely relevant in the maintenance of intracellular and extracellular homeostasis. [52]

The relationship between age-related eye diseases and nutrition with antioxidants has been known for some time, several herbal remedies have been used since the beginning of human civilization for treatments of night blindness, cataracts, floaters, or glaucoma. [55] Their effectiveness, however, was merely experimental, varying significantly due to nonstandard sources. Numerous studies have been looking at the relationship between antioxidant action and the therapeutic and preventive benefits of herbal compounds. [56]

Nutritional therapy with phytochemical interventions shows promise in reversing the eye disorders course. These phytochemicals found in nature are rich in polyphenols, saponins, carotenoids, and vitamins. [57]

Polyphenols are naturally occurring chemical compounds with multiple phenol units and are divided into phenolic acids, anthocyanins, stilbenes, and flavonoids. These are known not only as antioxidants but also as having anti-inflammatory properties along with other beneficial effects. [17], [57] Recent research has revealed some ocular health advantages of polyphenol consumption namely lowering lens opacity, reducing apoptosis in the RPE, and inhibiting blood-retinal barrier breakdown. [57] There is also evidence of the favorable impact of anthocyanins, which were associated with improvements in night vision. [58]

The mechanism of action of polyphenols in ocular diseases is focused on their ability to inhibit oxidative stress. [57] There are several compounds with known activity. Curcumin (diferuloylmethane) and turmeric treatment have been shown to reverse abnormalities in lipid peroxidation, increase GSH, activate antioxidant enzymes, and counteract hyperglycemia-induced oxidative stress. [57] Resveratrol (trans-3,5,4’-trihydroxystilbene), present in grapes, wine, and fruit berries, has been correlated with the reduction in the formation of the pigments such as lipofuscin and reducing glaucoma markers

expression, as well as, a significant antiapoptotic effect. [59], [60] There is also evidence that resveratrol causes an increase in GSH levels protecting the lens from ROS and preventing cataract formation. [59] In the retina, this compound was found to promote SOD activity not only in this tissue but as well in the blood of diabetic rats, preventing several ocular chronic damages resulting from diabetes. Furthermore, there was a decrease in endoplasmic reticulum stress in macular degeneration and the avoidance of apoptosis of human RPE cells. This compound also presents anti-tumorigenic activity in ocular tissues. [59]

Still, in the polyphenol category, there is rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside), a flavonoid present in citrus fruits such as lime, oranges, lemons, and grapefruits, and reported to scavenge free radicals. Rutin showed that effectively reduces retinal ganglion cell loss, and decreases intraocular pressure being a compound of interest for glaucoma treatment. [61] In another study, it was observed rutin's anticataractogenic effect, possibly by preventing the depletion of antioxidant enzymes and GSH, as well as by inhibiting lipid peroxidation. [62]

Carotenoids are a class of lipo-soluble pigments that include hundreds of natural compounds that are divided into two major categories: orange pigments called carotenes and yellow pigments called xanthophylls. The latter, particularly lutein, zeaxanthin, and meso-zeaxanthin, have a strong correlation with the antioxidant defense system, especially in the macula, where they are accumulated. It is worth noting that although there are more than 30 carotenoids in human blood, only these three are found in the most important area of the retina, probably explained by their disposition and behavior in retinal membranes. [63]–[65] These xanthophylls are accumulated in the region of photoreceptor axons and photoreceptor outer segments of the fovea, macula's center, in concentrations of up to 1000 times higher than in other tissues of the organism. [64] Carotenoids are extremely potent antioxidants that scavenge and neutralize free radicals including hydroxyl radicals and superoxide anion in photoreceptor cells, and also present anti-inflammatory, blue light filter out ability and functional and structural enhancement of synaptic membranes in the nerve system. [17], [63] The capacity of macular carotenoids to counteract oxidation processes in photoreceptor cells accounts for the relationship between carotenoid supplementation and a decreased prevalence of ocular disorders such as cataracts and AMD. [55], [65], [66] Another important aspect is the reduction of lipofuscin's formation in RPE due to carotenoids by free radical quenching. [67] Lutein and zeaxanthin also protect the eye from photooxidative damage and blue light thus protecting the lens and retina. [17], [64], [65]

Besides the antioxidants discussed in this section, there is also important to mention the amino-sulfonic acid taurine. It is present in various tissues of the eye namely, the retina, vitreous, lens, cornea, iris, and ciliary body, being the most concentrated in every ocular tissue when compared with other amino acids. [68] This compound has been studied due to its antioxidant properties as well as its potential to increase mitochondrial function by stabilizing the electron transport chain and reducing ROS formation. [69], [70] Some of its ocular benefits include increased retinal photoreceptor survival, and also promotes corneal wound healing, protecting corneal stroma and epithelium from lactic acidosis caused by contact lens wear, protecting ocular surface tissues from chemical damage, protecting diabetic lenses against

cataracts, reduces ocular inflammation, and induces a regenerative effect on contact lens wearers' tear film. [70]

Having these properties in consideration and the fact that most of them are insoluble or poorly water-soluble, [71], [72] it is important to find a delivery system that has that ability to be possible to utilize these antioxidants in ocular formulations topically administered. A solution can be through a drug system that can solubilize these poor soluble compounds and do it by the use of natural components which makes it safer, biocompatible, and also appealing to the pharmaceutical industry. One example of such a drug delivery system is the so-called deep eutectic systems (DES), which will be discussed in more detail in the next section.

1.8 Deep Eutectic Systems

DES are a recent class of alternative solvents and are defined as a combination of two or more components at a specific molar ratio that can establish hydrogen bond interactions with each other, thus presenting a large depression of the melting temperature when compared to the melting temperatures of each component individually. (Figure 1.5) This behavior can be credited to charge delocalization resulting from the hydrogen bonding between at least one hydrogen bond acceptor (HBA) and one hydrogen bond donor (HBD). [73], [74]

DES were first mentioned by Abbott, *et al* [75] in 2003 to describe the formation of a liquid eutectic mixture with an abnormal low melting point, starting from two solid materials (choline chloride:urea, 1:2) with high melting points in a specific ratio. The freezing point of this eutectic is 12 ℃, which is categorically lower than that of choline chloride and urea individually (choline chloride is 302 ℃ and urea is 133 ℃).

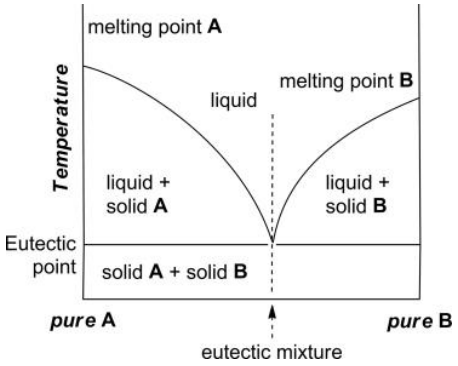

Figure 1.5 - DES phase diagram for a binary system [74].

A new identity with distinct physical and chemical qualities from the individual compounds is created because of interactions between the HBA and the HBD during the production of the DES. The selected molar ratio, entropy changes brought on by the intermolecular configurations, and starting components

all play a significant role in this. The physical characteristics of the DES, including viscosity, thermal behavior, density, polarity, and conductivity, are also greatly influenced by the composition. [76]

DES formation does not involve a chemical reaction. Hence, it does not need additional solvents, becoming a promising alternative to organic solvents that are normally used. [77] There is no need for purification steps thus being an option economically viable having into consideration the conventional solvents. These systems are considered the new generation of green solvents with multiple advantages namely, simple preparation, low cost of the raw compounds, and high biodegradability. [73], [78] The variety of elements and their arrangements leads to millions of combinations. [77] The fact that the individual components are well toxicologically characterized helps in the creation of large-scale projects. However, known individuals do not always mean knowledge of the system. Cellular toxicity is generally low but highly dependent on the composition, viscosity, and concentration of the DES. [73], [79] Biodegradability is a big issue when dealing with novel solvents. Based on the biodegradability and environmental effect of the individual components of DES, it is expected that these solvents are more biodegradable and have a lower environmental impact than other conventional solvents. This question was accessed by Hou, *et al* [80] observing the potential of several microbes to decompose DES by biodegradation, particularly anaerobic degradation. Most of the compounds tested degraded by up to 80% after 21 days.

Choline chloride-based DES are among the most common systems. [73], [81] From these natural deep eutectic systems (NADES) have emerged, which are eutectic mixtures of metabolites found naturally in certain types of organisms. They were presented by Choi, *et al* [82] when studying compounds present in the intracellular media but which were neither soluble in aqueous nor lipidic phases. They can be found in cells in biological processes and work as a third phase in organisms to facilitate the transport of hydrophilic, lipophilic, and poorly water-soluble compounds. It was suggested that NADES are also involved in the biosynthesis, solubilization, and storage of numerous hydrophobic metabolites and unstable compounds in cells. [83] Likewise, several biological pathways for drought resistance, dehydration, germination, and cryoprotection have been linked to NADES. [84]
NADES include primary and secondary metabolites such as organic acids, amino acids, sugars, or choline derivatives and frequently include water in certain molar ratios. [78], [83], [85] NADES similarly to DES have a great variety of physicochemical characteristics, namely viscosity, conductivity, density, and polarity, and all depend on each system, and their individual elements, even more, when metabolites can have such a range of polarity, ratios, and their intermolecular interactions and the water contribution. Also, these systems are usually liquid at room temperature, with some systems even below 0 °C. Dai, *et al* [83] investigated its decomposition and observed that when heated to 100 °C for 1 hour, the systems tested (where the system Fructose:glucose:sucrose:water (1:1:1:11) is common to the list of this work) showed with no discernible decomposition and the tested systems with sugars in their composition only began to decompose at 135 °C. Viscosity is one of the most important features, as well as one of the most significant barriers to DES implementation. Their values vary with water concentration with huge depressions with low water concentrations, yet it maintains its characteristics. In terms of polarity, there are several ranges, where the organic acid-based DES, as the ones containing

malic acid, are the most polar, and those based on sugars and polyalcohols presented to be the least polar. This characteristic makes them interesting alternatives to conventional solvents due to its wide range physicochemical properties. These can be tailored with the addition of water, lowering viscosity and density, and increasing their polarity. However, water quantities above 30% in some cases, to around 50% cause intermolecular and structural disruptions. [83], [85], [86]
Another interesting feature of the NADES is the fact that these solvents can form additional hydrogen bonds with solutes and this characteristic opens an infinite world of applications, as it can contribute to the stabilization of sensitive molecules. NADES can dissolve non-water-soluble metabolites even with small amounts of water. Furthermore, the temperature has a significant impact on the solubility of compounds in NADES. It is well documented that macromolecules such as proteins, polysaccharides, and DNA are soluble in NADES. [83]
Some of the applications of DES already described in the literature include the metallurgy industry, separation, and gas capture, power systems, biological catalysis, organic chemistry, biomass processing, biomolecular structure, folding, and stability knowledge, genomics, nanomaterials synthesis, pharmaceutical, and medical research. [77]

NADES have been considered for several purposes in combination with antioxidants. Dai *et al* [87] tested NADES as an extraction solvent of phenolic compounds from the safflower in comparison with water and ethanol, where NADES showed higher extractability of phenolic compounds, both polar and less polar. The water content in these systems resulted in a decrease in viscosity and increased yields for polar compounds, and the opposite effect occurred for the extraction of less polar phenols, being important in the optimization of these parameters when performing extractions with NADES. The physical properties mentioned above show that NADES, being a green alternative, can be employed in natural product extractions for pharmaceutical usage.

In another study, Dai *et al* [83] determined the solubility of rutin, which is up to 100 times higher than water. Additionally, the NADES with the highest quantities of water (10% v/v) was the one that afforded the best results. These results showed that water is an important factor in optimizing natural NADES. Faggian *et al* [88] also solubilized rutin in NADES in quantities high as twenty times when compared with water, and its absorption and elimination from plasma were quite fast. This leads to the conclusion that NADES improved bioavailability by solubilizing higher quantities of compounds promoting these systems as a good chance in pharmaceutical applications.

Likewise, DES also started to be used not only as a delivery system but as well as a bioactive system, having active pharmaceutical ingredients (API) as components, emerging then therapeutic deep eutectic systems (THEDES), which have been characterized by having anti-fungal, anti-bacterial, anti-viral and anticancer activities with increased bioavailability and permeation. [89] Having this in consideration, it is observed that eutectic mixtures not only present pharmaceutical activity but are also able to be a good candidate to be evaluated as an ocular drug delivery system, by mainly increasing bioavailability.

1.9 Objective/Aim

The main aim of this thesis is to develop an ocular delivery system using NADES, that can carry compounds with antioxidant activity to prevent the most critical eye diseases. For that, several systems were chosen including sugars, amino acids, and phenols combined as matrixes for further solubilization of bioactive compounds, namely antioxidants. It was tested different systems from those reported in the previous section because choline chloride, is a compound banned for cosmetic applications according to Regulation 1223/2009 (entry no. 168: choline salts and their esters, e.g. choline chloride) [90] and in pharmaceutical application it was nominated to be used as an API only in oral and intravenously.[91] Lactic acid was also discarded due to probable toxicity in ocular cells. Relatively to the second article, the best system was Proline:Glutamic acid (2:1), but despite glutamic acid is one of the most abundant amino acids found in ocular tissues after taurine, [68] it was decided to exclude due to its acidity. Nevertheless, it is a great system for further exploration.

To ensure that NADES can be used as ocular delivery systems by topical instillation several tests were performed on their physicochemical properties namely, viscosity, osmolality, pH, refractive index, density, surface tension, and contact angle. Additionally, the systems were evaluated in terms of their cytotoxicity to understand their effect on the ocular environment.

Finally, it was also performed a stability assay, coming from previous work, to assess the antioxidant activity by DPPH assay over a 6 month period of some systems with antioxidants namely rutin, taurine and resveratrol solubilized.

2. MATERIALS AND METHODS

2.1 Preparation of NADES

Betaine anhydrous (≥99%, Sigma), trehalose dihydrate (Hashibara, Japan), Glycerol (99.5%, Scharlau), Ethylene Glycol (≥99,5%, Carlo Erba), D-(+)-glucose anhydrous (≥97,5%, Farma-Química), D-(-)Fructose (Sigma), D(+)-Sucrose (99.5%, Sigma), L-Proline (99%, Alfa Aesar), *N*-Acetylcysteine, (Sigma), D-Sorbitol (98%, Sigma-Aldrich).

Different NADES were prepared using these components at these particular molar ratios by slowly mixing and heating the mixture between 35 and 60 ℃, with constant stirring, until a clear liquid was obtained. [92] The water content of the NADES was determined using an 831 KF Coulometer (Metrohm) with a generator electrode and without a diaphragm. The water content values presented in Table 2.1 are an average of three measurements and a conversion from ppm to percentage.

2.2 Eye drop formulation

NADES were diluted in an artificial tears solution (AT) - 0.9% NaCl (PanReac AppliChem) - at 5 and 10 % w/w concentration (approx. 5.3 and 10.5 mg/mL) to perform viscosity and osmolality analysis.
To perform pH, density, surface tension, contact angle, and refractive index, NADES were diluted in phosphate-buffered saline (PBS, Sigma) at 5 and 10% w/v (5 and 10 mg/mL) concentration.

2.3 Viscosity

The rheology studies of the different systems were evaluated using an Anton Paar Modular Compact Rheometer 102 fitted with parallel plate geometry with a 49.954 mm diameter (PP50, Anton Paar) and 0.5 mm gap. Before each measure samples were trimmed and then stabilized.

The viscosity as a function of temperature measurements were performed after 5 minutes of stabilization at 15℃ under a constant shear rate of 10 s^{-1} and between 15 and 40 ℃ at a rate of 1.1 ℃/min. The data is represented as the average of three measurements.

The viscosity as a function of shear rate measurements were performed after 5 minutes of stabilization at 0 s^{-1} under a constant temperature of 25 ℃ and between 0 and 100 s^{-1} at a rate of 4.35 s^{-1}/min. The data is represented as the average of three measurements.
The same evaluation was executed with a 0.3% (w/v) HPMC solution in PBS and with Tobrex® which were used as a comparison.

Table 2.1 - Preparation of NADES.

NADES	Components				Molar Ratio	Water Content (%)
	A	B	C	D		
Bet:Treh:W	Betaine	Trehalose	Water	-	4:1:10	21.8 ± 0.7
Bet:Treh:Gly:W	Betaine	Trehalose	Glycerol	Water	2:1:3:5	13.6 ± 0.1
Treh:Glc:Gly:W	Trehalose	Glucose	Glycerol	Water	1:2:2:3	11.2 ± 0.2
Bet:EG	Betaine	Ethylene Glycol	-	-	1:3	0.8 ± 0.1
Bet:Glc:W	Betaine	Glucose	Water	-	5:2:12	16.4 ± 0.1
Fru:Glc:Suc:W	Fructose	Glucose	Sucrose	Water	1:1:1:11	19.4 ± 2.4
Glc:Pro:Gly:W	Glucose	Proline	Glycerol	Water	3:5:3:20	20.1 ± 0.6
Bet:Suc:Gly:W	Betaine	Sucrose	Glycerol	Water	2:1:3:5	10.0 ± 0.1
Bet:Gly	Betaine	Glycerol	-	-	1:2	1.7 ± 0.1
Bet:Pro:W	Betaine	Proline	Water	-	1:2:10	12.9 ± 2.9
Bet:NAC:W	Betaine	*N*-Acetyl Cysteine	Water	-	1:1:3	17.7 ± 1.1
Bet:Sorb:W	Betaine	Sorbitol	Water	-	1:1:3	13.8 ± 1.5
Bet:Suc:W	Betaine	Sucrose	Water	-	4:1:10	16.7 ± 0.4
Bet:Suc:Pro:W	Betaine	Sucrose	Proline	Water	5:2:2:21	18.5 ± 0.3
Gly:Glc	Glycerol	Glucose	-	-	4:1	0.2 ± 0.1

2.4 Osmolality

This analysis was performed using a KNAUER Freezing Point Osmometer K-7400S. Osmometer measurement ranges from 0 to 2000 mOsmol/kg with a resolution of 1 mOsmol/kg. The calibration curve was performed using water (Carlo Erba Reagents, HPLC plus) as the 0 mOsmol/kg and supplied solutions (400 and 850 mOsmol/kg). The values presented are an average of three measurements.

2.5 pH

The measurements were performed using a Methrom 914 pH/Conductometer with 0.001 pH of resolution. The values presented are an average of three measurements. pH-meter was calibrated with three standard buffered solutions of 4.00, 7.00, and 10.00 pH (Fluka). All measurements were performed at room temperature.

2.6 Density

The measurements were performed with an Anton Paar Stabinger Viscometer 3001 from 20 to 60 °C with an increase of 10 °C/point. Each value results from an average of three measurements.

2.7 Surface tension

The measurements were performed using a standalone force tensiometer (Biolin Scientific Sigma 702) with a Du Noüy ring using the Huh-Mason correction. The values given are an average resulting from three consecutive measurements at 25 ºC, obtained from the temperature controller RW-0525G Lab Companion.

2.8 Contact Angles

The contact angle acquisition was performed using an optical goniometer (CAM 100, KSV Instruments Ltd) that captured the drop image, and the respective software, CAM 100, calculated the value of the contact angle from it based on the width and height of the drop. For each drop, 10 frames were captured with 1000 ms of an interval between them. The contact angle values are an average of three

measurements. The method of contact angle measurement was the sessile drop in a polytetrafluoroethylene (PTFE) surface.

2.9 Refractive Index

The refractive index measurements were performed at room temperature, using a monochromatic Abbe-2WAJ Refractometer. Each measurement was repeated three times.

2.10 *In vitro* cytotoxicity assessment

These studies were performed using the L929 (DSMZ - German Collection of Microorganisms and cell culture GmbH) which are mouse fibroblasts approved by ISO biocompatibility procedures - ISO 10993-5:2009 and in ARPE-19 cell line (ATCC) which is an immortalized human spontaneously arising retinal pigment epithelia (RPE).

2.10.1 Cells thawing

The cryovial was warmed up in the 37 °C water bath for a few minutes and then transferred to a falcon tube containing warmed complete media and centrifuged at 200 rcf (or g) for 10 min. Then, the supernatant was discarded by inversion, because some cryoprotectants like the one here used, DMSO, are toxic at temperatures higher than 4 °C, and cells were resuspended in 1 mL of cell culture medium and transferred to a 25 cm^2 culture flask (Corning).

2.10.2 Cell culture

ARPE-19 cells were maintained in Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham with L-glutamine, 15 mM HEPES, and sodium bicarbonate, (DMEM F-12, Sigma-Aldrich), supplemented with 10% (v/v) of fetal bovine serum (FBS, Corning) and 1% (v/v) of penicillin-streptomycin (PS, Corning). L929 were grown in Eagle's Minimum Essential Medium (MEM, Corning) supplemented with 10% FBS (Corning) and 1% PS (Corning).

Cell cultures were grown in 75 cm^2 culture flasks (Falcon) until they reached 80-90% of optical confluence in a humidified atmosphere, at 37 °C with 5% of CO_2.

2.10.3 Cell viability

ARPE-19 cells were detached from the T-flask through the addition of accutase (Corning) and then incubated for 24 hours in a 96-well plate at a density of 1.0 × 10^4 cells/well. For L929 cells, the same process was executed but the detachment agent was trypsin (Corning). Afterward, each NADES was added at 5% and 10% (w/v) concentration and incubated for one day at 37 °C and 5% CO_2. Control cells were incubated only with complete media. To evaluate cell viability, it was used CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega), based on MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which is a reduction reaction of MTS that cells with active metabolism perform based on dehydrogenase enzymes, into a colored formazan product. (Figure 2.1)

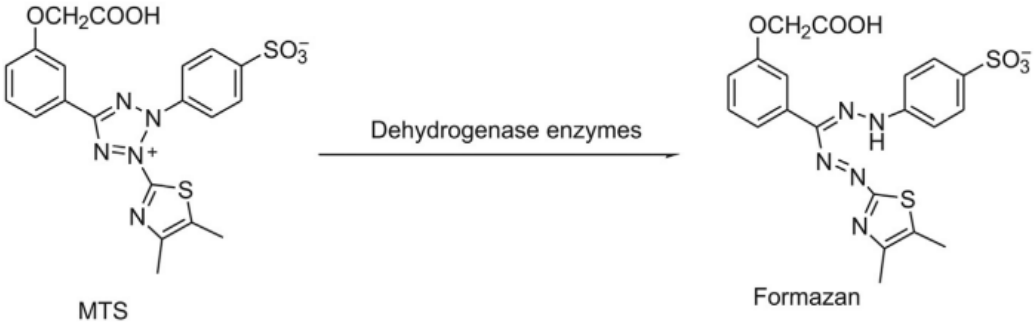

Figure 2.1 - Mechanism of action of MTS assay [93]

After 24 hours of incubation, the cells were washed with PBS (Sigma), and then the MTS was added and incubated for 2 hours at 37 ºC before reading its absorbance at 490 nm in a microplate reader (VICTOR Nivo™, PerkinElmer), where absorbance is proportional to the quantity of viable cells in the culture (Eq.1). Cell viability was then represented as a percentage in comparison to the control cells. Each NADES was tested in triplicate.

$$\%Cell\ viability\ = \frac{A_{490nm}sample}{A_{490nm}control} \times 100 \qquad \text{(Eq. 1)}$$

2.11 Antioxidant Activity stability

In a previous work carried out in the research group, three antioxidants, rutin hydrate (94%, Sigma), taurine (99%, Sigma), and *trans*-resveratrol (99%, Sigma) were dissolved in different NADES as shown in Table 2.2. [94], [95] In the current work, the stability of those antioxidants in NADES was assessed at predetermined timepoints and in the same concentrations used throughout these works. These samples were stored at room temperature between 20 and 25 ºC controlled by air conditioner.

Table 2.2 - Antioxidant concentration in NADES

Antioxidant	**NADES**	**Concentration (%, w/w)**
Taurine	Bet:Treh:W	0.1; 0.25; 0.5; 1.0
	Bet:Treh:Gly:W	0.1; 0.5; 1.0
Resveratrol	Bet:Treh:W	0.1; 0.25; 0.5; 1.0
	Bet:Treh:Gly:W	0.1; 0.25; 0.5; 1.0
Rutin	Bet:Treh:W	0.1; 0.2; 0.25; 0.5; 1.0; 2.5
	Bet:Treh:Gly:W	0.05; 0.075; 0.1
	Bet:Eg	0.1; 0.2; 0.25; 0.5; 1; 2.5

The antioxidant activity was determined through the DPPH assay. This method uses a free radical, characterized by its deep-violet color, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), which is soluble in methanol and presents an absorption maximum at 517 nm. Antioxidants and other radical species react with this stable radical (DPPH·) by providing an electron or hydrogen atom, hence reducing the radical to 2,2-diphenyl-1-hydrazine (DPPH-H)) characterized by pale-yellow color (Figure 2.2) which is can be followed by a UV-VIS spectrophotometer at the same wavelength. [96]

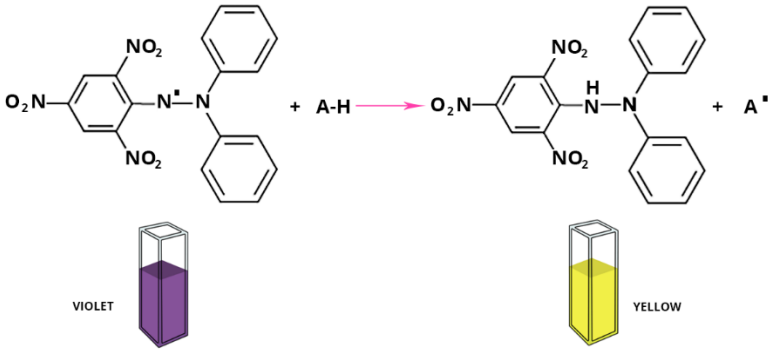

Figure 2.2 - DPPH assay chemical basis [97]

A 24 % (w/v) stock solution of DPPH was prepared in methanol (≥ 99.8% Honeywell) and stored at 20 ºC until necessary. Then, a new solution was prepared by dilution, becoming a solution of 8% (w/v) with methanol with some adjustments to obtain an absorbance at 517 nm with values between 1.000 and 1.100. Next, in a microplate, 200 µL of this solution was added to 7.5 µL of the samples or water (control).. After it was left reacting in the dark for 40 minutes and the absorbance read at 517 nm in a

microplate reader (VICTOR Nivo™, PerkinElmer). The determination of DPPH radicals scavenging activity (RSA) is obtained according to the following equation:

$$\%RSA = \frac{A_{517nm}control - A_{517nm}sample}{A_{517nm}control} \times 100 \quad \text{(Eq. 2)}$$

For each sample and the control, triplicates were used, and the values represented in the graphs are the resulting mean and SD. Water was used to simulate the 0% of RSA.

2.12 Statistical Analysis

The statistical analysis was carried out using the software GraphPad Prism 8.0.1 (GraphPad Software). The data presented is expressed as mean ± Standard Deviation (SD) and significant differences were calculated in comparison of the different solutions with the control. It was considered statistically significant p-values smaller than 0.05. To perform comparison was performed two-way ANOVA following Tukey multiple comparison test. Statistical differences are represented by an asterisk.

3. RESULTS AND DISCUSSION

To select the working NADES concentrations some previous data was revised. Particularly relevant is the cytotoxicity of the systems. Jesus *et al.* [98] observed the cytotoxic effects of different sugar-based NADES in L929 cells, a cell line that is frequently utilized for cytocompatibility studies. The findings showed that cells can withstand high concentrations without losing viability. All systems presented a different behavior, but high cell viability, which is above 90 %, was obtained when cells were tested with 5% (w/v) of NADES, being well tolerated in concentrations of 10% (w/v). At 20% (w/v) of NADES, there was a significant disruption in cell viability. Having these results into consideration, it was decided to use the NADES presented in Table 2.1 at 5% and 10% (w/v).

3.1 NADES cytotoxicity evaluation

Likewise, in this work, it was performed an initial screening to observe the NADES effect on cells. Having information on cytotoxicity, it can be chosen the best systems to go forward, and at which concentrations. At first, it was evaluated cytotoxicity in L929 cells, followed by a specific analysis in ARPE-19 cells.

3.1.1 In L929 cells

Similarly to the study mentioned before [98], using the MTS assay, some of the NADES in Table 2.1 were tested in L929 cells, at 5 and 10 % (w/v) as can be seen in Figure 3.1. This was performed in an early stage of the project, where NADES list was not yet complete, which was not an issue, as this was a screening test while ARPE-19 cells were not at our disposal. Later, once ARPE-19 became available, detailed cytotoxicity studies were performed in this cell line because it is a more relevant cell line to assess this study

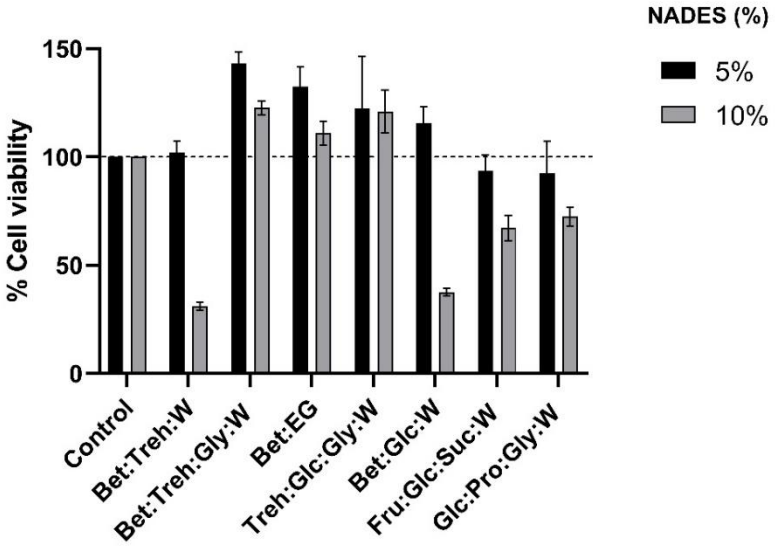

Figure 3.1 – Cell viability on L929 cells with different concentrations of NADES. Data indicated as mean + SD

NADES at 5% (w/v) concentration presented viability close to or above 100%. As expected, at higher concentrations, there was a slight decrease in cell viability, but interestingly, with certain systems namely Bet:Treh:Gly:W, Bet:EG, and Treh:Glc:Gly:W no cytotoxic effects were observed. These results helped to anticipate the NADES behavior in ARPE-19 cells at the desired concentrations.

3.1.2 In ARPE-19 cells

After the first screen, the same evaluation was performed in ARPE-19 cells, where all NADES from Table 2.1 were tested at both concentrations. (Figure 3.2).

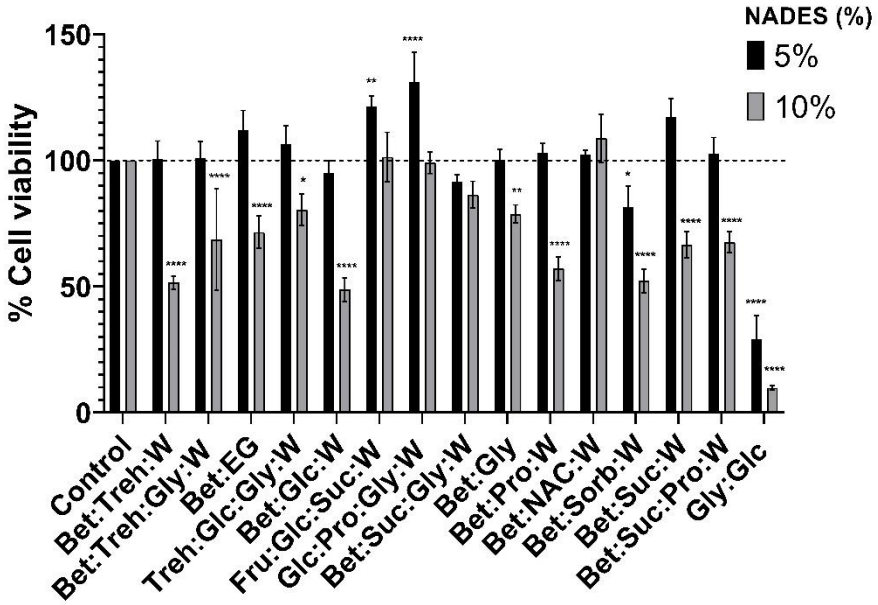

Figure 3.2 – Cell viability on ARPE-19 cells after 24 hours of incubation with different concentrations of NADES. Data represents mean ± SD (n = 3). Statistically significant differences were determined by Tukey's multiple comparisons test and are represented in asterisks: *p < 0.05; ** p < 0.01; **** p < 0.0001, two-way ANOVA. The absence of asterisks means that there are no significant differences when compared with control values at both concentrations.

The systems that were previously tested in L929 cells presented similar results in ARPE-19 cells, with the same systems showing increased cytotoxicity at 10% concentration. Furthermore, when compared with Jesus *et al.* [98] results, the system Gly:Glc is the one that presented different outcomes between cell lines, being highly toxic in ARPE-19 cells.

Overall, the remaining systems showed no significant cytotoxicity at 5% (w/v) concentration except for Bet:Sorb:W which presents values around 80% of cell viability, which is also not a great value in this matter. At 10% the systems Fru:Glc:Suc:W, Glc:Pro:Gly:W, Bet:Suc:Gly:W, and Bet:NAC:W presented cell viabilities around or above 100%. The remaining systems work significantly worse, presenting values highly below 100%.

3.2 Physicochemical properties of NADES formulations

3.2.1 Rheology studies

As discussed in section 1.5 viscosity is a crucial property of topical eye drops to achieve longer precorneal residence time. High viscosities might cause pain, discomfort, and eye injury. The goal is to find a reasonable gap between an improved retention time and the occurrence of adverse effects.

Shear rate is an essential factor to consider when working with rheology and is defined as velocity gradient. The viscosity of certain fluids, such as water or mineral oil, is independent of the shear rate used. These are referred to as Newtonian fluids. Other compounds have a viscosity that decreases as the shear rate rises, a property called shear-thinning. [99] Ocular surface shear conditions range from essentially at rest, when the eye is immobile, with values of 0.03 - 0.14 s^{-1}, to significant shears imposed while blinking. An eye drop should have a high viscosity at a low shear rate. At higher shear rates, associated with eye blinking, values are estimated to vary between 4000 and 28000 s^{-1}. [100] An eye drop viscosity approaching that of natural tears helps to reduce blurring and significant discomfort when blinking. [101]

Human tears viscosity values were assessed throughout several shear rates. Tiffany *et al.* [100] studied human tear samples in a shear rate range of 2 - 160 s^{-1}. All samples exhibited significant shear-thinning, with viscosity declining from around 5 mPa.s at the lowest shear rate to about 1.5 mPa.s at the maximum shear rate. Gouveia *et al.* [11] obtained the highest value of viscosity of 2.33 mPa.s at a shear rate of 0.0175 s^{-1} and, at the highest shear rate, 128.5 s^{-1}, the viscosity value was 0.97 mPa.s. The latter also assessed that the tear film without the lipid layer has a Newtonian behavior with a viscosity at a constant value of 1.0 mPa.s at all shear rates. [11]

NADES were also studied in terms of these rheological properties. Aroso *et al.* [102], [103] have tested several systems and determined that at a high shear rate, the flow behavior of the tested NADES systems is temperature and shear rate independent. Altamash *et al.* [104], [105] studied the effect of shear rate on the apparent viscosity at different temperatures. In this work, it was observed shear-thinning effect where the viscosity decreased with shear rate at all temperatures, concluding that NADES

behave like non-Newtonian liquids, similar to solid-like behavior. However, it must be considered that this shear-thinning occurred in shear rates below 1 s^{-1}, having no impact on higher values.

In this section, a commercial sample, Tobrex® Ophthalmic Solution (Tobramycin 0.3%, Novartis) was also used as a comparison. All eutectic systems from Table 2.1 were tested at 5 and 10% (w/w) concentrations. Here is a representative example, in Figure 3.3 and the remaining graphs present in Annex A.1. NADES solutions were prepared using a solution of 0.9% (w/v) of sodium chloride in PBS mimicking artificial tears (AT) at the eye surface.

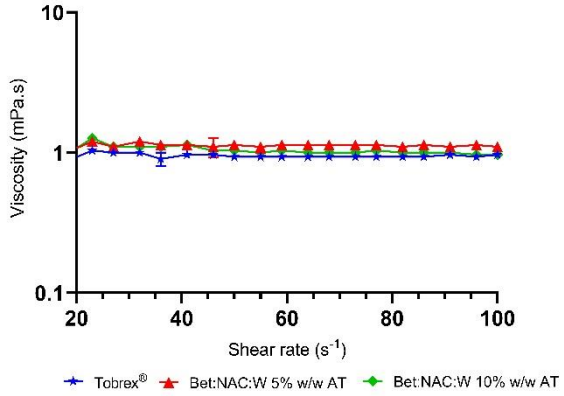

Figure 3.3 – NADES Bet:NAC:W viscosity as a function of shear rate, at 25 ºC in comparison with Tobrex®. Data indicated as mean + SD

These formulations (Figure 3.3 and Figure A.1 found in the Annex) exhibited newtonian behavior, with the viscosity being constant and independent of the shear rate applied. The readings were not precise at shear rates lower than 20 s^{-1}, presenting a high decrease of viscosity, similar to shear-thinning, but then it stabilized at 20 s^{-1}, most likely due to some inaccuracy in stabilization or liquid spread, resulting in variation in viscosity values in the measurements at those shear rates. An explanation can be due to non-ideal experimental conditions, namely fluid inertia, secondary flows, surface tension, slip at the boundaries, or sample underfill or overfill. Non-ideal conditions can result in misinterpretations of results, such as the observation of apparent shear-thinning and shear-thickening for a fluid that is newtonian. [106] In these measurements, NADES solutions also apparently looked to have a shear-thinning behavior in shear rates lower than 20 s^{-1}. Ewoldt *et al.* [106] presented similar experiments, where newtonian fluids presented shear-thinning at low rates and shear-thickening at high rates. In these cases, it is important to find the experimental window to obtain accurate results. This behavior often occurs when the samples are biologically complex, in fluids that have low viscosity similar to these NADES solutions and can be surface active components that modify the interface of the sample with the air. In this case, as trimming was performed before the 5-minute stabilization, the latter explanation may be the most probable. In addition, Johnston *et al*. [107] also observed the same behavior and in a similar way, concluded by assigning this observed effect to surface tension, which may be misinterpreted as shear viscosity thinning as the conclusion that was initially given in this evaluation as well.

The viscosity values at a shear rate above 20 s^{-1} were similar or slightly higher than Tobrex®'s and in the same range of values as human tears, whereas at the higher shear rates NADES' viscosity ranged between 0.8 mPa.s and 1.2 mPa.s. Taking into consideration the information mentioned in section 1.5, these values indicate that these NADES formulations alone are unlikely to improve precorneal residence time. Nevertheless, it was not possible to increase the concentration of NADES without compromising the toxicity and other adverse effects. Therefore, it might be necessary to use a viscosity enhancer, such as HPMC or others mentioned in section 1.5, but at a lower concentration than is generally used in commercial samples.

Analyzing the same graphs, the systems present similar values in this shear rate range. At the highest shear rate measured, all solutions had viscosities between 0.8 and 1.1 mPa.s The highest NADES formulations are Bet:NAC:W 5% (w/w) AT, Treh:Glc:Gly:W 10% (w/w) AT, Glc:Pro:Gly:W 10% (w/w) AT and Gly:Glc 10% (w/w) AT, all at 1.1 mPa.s. Nevertheless, the differences between all formulations are small, being barely irrelevant when compared with other ocular formulations.

To assess the behavior and viscosity values of eye drops already available in the market, Che Arif *et al.* [108] tested eighteen commercial artificial drops at 100 s^{-1} of shear rate. The highest viscosity value was 34.39 cP (Vismed® Gel, TRB Chemedica) and the lowest was 0.55 cP (Cationorm®, Santen) with all exhibiting shear-thinning tendency. Here the lowest eye drop is lower than NADES solutions but has values far away from the highest viscosity values. Kapadia *et al.* [109] measured the shear viscosities of twelve commercial eye drops at three different temperatures, and shear-thinning behavior was seen in each test. The most interesting conclusion from this study was that at low shear rates, especially when the eye is opened, people can benefit from high viscosity in artificial tears, but then at low shear rates, eye drops with low viscosities evaporate rapidly and are susceptible to rapid drainage. However, at higher shear rates, such as while blinking, the viscosity of the eye drops is required to be lower as referred to in section 1.5, indicating that shear-thinning is the appropriate property for an ocular drop to take advantage of both stages. This conclusion implies that is important that these formulations act as non-Newtonian, thus it is necessary to transform or to add some compound that has or gives this behavior in NADES ocular formulation.

Nevertheless, there are tears with similar properties. Arshinoff *et al.* [110] tested 20 artificial tears commercially available. These tears were divided into three groups: those with considerable shear-thinning behavior, those with moderate shear-thinning behavior, and those that exhibit newtonian behavior. This third group had viscosities ranging from 1 mPa.s to 12.30 mPa.s A noteworthy detail of the commercial samples of this group of newtonians, is that half of them contained trehalose as an excipient, a compound present also in some tested NADES, which is a well-known disaccharide that has been linked to ocular protective qualities such as photooxidation and is implicated in oxidative, inflammatory, and apoptotic pathways in the cornea. [111]

Nevertheless, solutions with essentially equal or similar constituents can have substantially diverse rheological characteristics. [110] Yet, it is important to acknowledge that the majority of the newtonian eye drops were below the threshold of 10 mPa.s, causing retention time issues, which is similarly the

prominent issue that these NADES formulations present. However, it cannot be discarded by the utilization of these formulations, but it cannot be used itself.

Observing Figure 3.4 and Figure A.2 in the Annex, it is clear that viscosity is temperature dependent with the first decreasing as the temperature rises. The decreasing behavior is slightly different from Tobrex®'s, although viscosities were similar at physiological temperatures. NADES can have marginally higher viscosities at room temperature, though the difference is around 0.5 mPa.s. These measurements were obtained at low shear rates, meaning that this difference should continue to exist at higher levels.

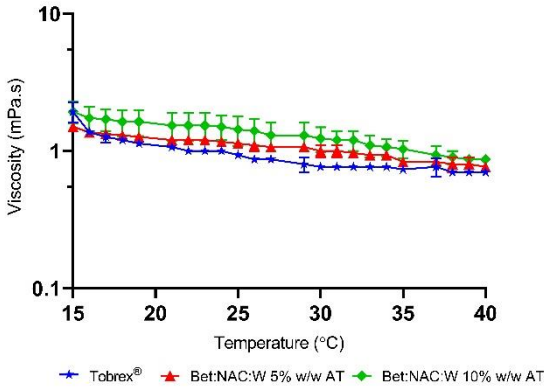

Figure 3.4 - NADES Bet:NAC:W viscosity as a function of temperature, at 10 s^{-1} in comparison with Tobrex®. Data indicated as mean + SD

The highest value of viscosity at 15 ºC belongs to Gly:Glc 10% (w/w) AT and at 40 ºC to Bet:NAC:W 10% (w/w) AT. At 5% (w/w) concentration, Bet:Suc:Gly:W has the highest viscosities. It is important to know NADES behavior at these temperatures to know which viscosities are expected and if the formulation is significantly affected, thus decreasing the bioavailability. Rahman *et al.* [44] added that the currently used ocular lubricants have issues in this matter due to reductions in viscosity by temperature and changes in dilution. The objective then is to develop eye drops with stable viscosities maximizing therapeutic efficacy. The dilution topic was also addressed in this project, using one system, Bet:Treh:Gly:W, with the figure shown in the Annex (Figure A.3) demonstrating the enormous viscosity decline with only a small amount of water, confirming the dilution concerns that are common to the majority of eye drops are also present in NADES formulations.

To understand the polymer behavior and to compare it to NADES formulations, it was also performed the same measurements explored in this section. The polymer used was HPMC, and the concentration tested as a comparison was 0.3% w/v, which is the concentration normally reported in commercial samples. Figure 3.5 shows that HPMC viscosity displays newtonian behavior as well but at higher values than NADES solutions. These results indicate that the polymer could promote longer retention times than these NADES formulations. Having these results in consideration, it would be interesting to determine if it is feasible to employ these polymers as excipients simultaneously with NADES.

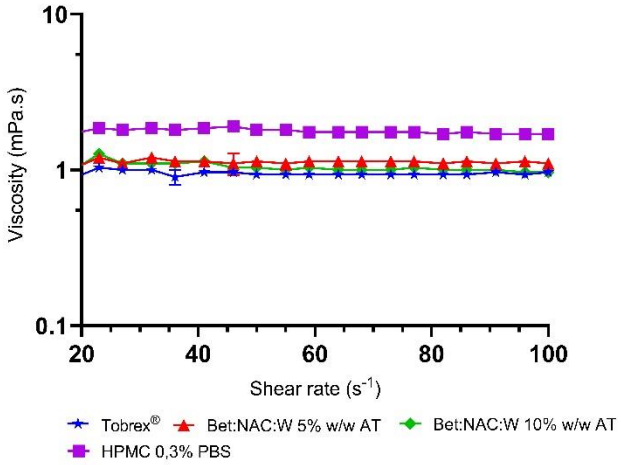

Figure 3.5 - NADES Bet:NAC:W viscosity as a function of shear rate, at 25 ºC in comparison with Tobrex® and HPMC 0.3% (w/v). Data indicated as mean + SD

3.2.2 Osmolality

The eye surface is in a constant dynamic of tears production, evaporation, absorption, and drainage. This can be addressed by one single property which is tear dynamics' final product: osmolality. [112] The systems from Table 2.1 were all measured by freezing point osmometry. Similarly to the previous analysis, it was used the AT solution, which has osmolality closer to the tear film. [113]

Table 3.1 - Osmolality values. NADES samples in AT. Data indicated as mean + SD

Sample	**Osmolality [mOsmkg^{-1}]**	
Commercial Sample - Tobrex®	214 ± 1	
Artificial Tears	286 ± 1	
HPMC 0.3% PBS	280 ± 1	
NADES	**5% w/w AT**	**10% w/w AT**
Bet:Treh:W 4:1:10	554 ± 1	857 ± 2
Bet:Treh:Gly:W 2:1:3:5	631 ± 3	1039 ± 4
Treh:Glc:Gly:W 1:2:2:3	473 ± 2	689 ± 1
Bet:EG 1:3	962 ± 3	1883 ± 1
Bet:Glc:W 5:2:12	602 ± 4	947 ± 3
Fru:Glc:Suc:W 1:1:1:11	412 ± 2	558 ± 2
Glc:Pro:Gly:W 3:5:3:20	538 ± 2	825 ± 1
Bet:Suc:Gly:W 2:1:3:5	639 ± 2	1032 ± 4
Bet:Gly 1:2	434 ± 4	1504 ± 4
Bet:Pro:W 1:2:10	587 ± 2	957 ± 1
Bet:NAC:W 1:1:3	563 ± 2	900 ± 4
Bet:Sorb:W 1:1:3	585 ± 1	922 ± 7
Bet:Suc:W 4:1:10	554 ± 2	875 ± 3
Bet:Suc:Pro:W 5:2:2:21	541 ± 5	833 ± 2
Gly:Glc 4:1	709 ± 3	1207 ± 8

As observed, all NADES formulations have higher osmolarities than the commercial sample. They can be considered hyperosmolar because human tears have an osmolality of 304 mOsmol/kg. [113] At both concentrations, the solutions that presented the lowest osmolality were using the Fru:Glc:Suc:W system. Generally, systems with glycerol had increased osmolalities, when compared with similar systems without this compound, Bet:Gly 5% (w/w) AT is an exception.

To mention that this method is known for its inaccuracy in measuring fluids with high viscosity [114], which occurs with some of these formulations tested, visible for example on the system Bet:Treh:Gly:W which at 30% in AT had an osmolality of 3648 ± 147 mOsmol/kg, which is already outside of the limit of quantification of the osmometer utilized.

Dutescu *et al.* [114] tested the osmolarity of 87 commercially available eye drops and their effects on the cornea. Interestingly, the distribution between hypoosmolar and hyperosmolar eye drops was barely the same. The lowest sample had 131 mOsmol/L and the highest had 1955 mOsmol/L showing an enormous disparity between the samples. *In vitro* studies suggest that hypertonic drops can modify the tear osmolarity and consequently cause inflammation, however, the rapid clearance makes the predictions of the *in vivo* effects difficult. The authors concluded that more important than the actual value of osmolarity, the products used, and their cytotoxicity are far more relevant.

The fact that there are in the market eye drops with osmolarities as high as near 2000 mOsmol/L suggests that the osmolarity of NADES formulations, where the highest value was 1883 mOsmol/kg, does not pose a limitation for their use as a delivery system for ocular applications.

Hyperosmolality of ophthalmic formulations can cause some issues when dealing with people suffering from DED, or that are susceptible to it, however, as the purpose is to utilize these formulations as a preventative measure, this is not a major concern. Nevertheless, Cabral's research [95], revealed that NADES with rutin added had lower osmolarities than NADES in pure form, whereas in Suyarko's [94] the same behavior was not observed, having similar osmolalities after the addition of the antioxidants, which leads to the conclusion that it is important to access all future components, including the antioxidant, before reaching an osmolality conclusion.

3.2.3 pH

Depending on their composition NADES formulations can significantly affect the pH of aqueous solutions. All systems in Table 2.1 were evaluated to see their effect on the pH on the PBS solution (pH 7.42). The pH value of eye drops should ideally be near 7.4 – 7.6 [115], [116], which resembles the tear film, hence the utilization of PBS buffer as a solvent to perform this study.

Table 3.2 - pH values of NADES samples in PBS

Sample	**pH**	
Commercial Sample - Tobrex®	7.47	
PBS	7.42	
NADES	**5% w/v PBS**	**10% w/v PBS**
Bet:Treh:W 4:1:10	7.47	7.50
Bet:Treh:Gly:W 2:1:3:5	7.40	7.40
Treh:Glc:Gly:W 1:2:2:3	7.34	7.29
Bet:EG 1:3	7.43	7.52
Bet:Glc:W 5:2:12	7.40	7.43
Fru:Glc:Suc:W 1:1:1:11	7.32	7.29
Glc:Pro:Gly:W 3:5:3:20	7.28	7.20
Bet:Suc:Gly:W 2:1:3:5	7.37	7.37
Bet:Gly 1:2	7.46	7.42
Bet:Pro:W 1:2:10	7.38	7.33
Bet:NAC:W 1:1:3	2.65	2.64
Bet:Sorb:W 1:1:3	7.43	7.43
Bet:Suc:W 4:1:10	7.46	7.45
Bet:Suc:Pro:W 5:2:2:21	7.42	7.42
Gly:Glc 4:1	7.38	7.28

The pH values ranged from 7.20 to 7.52 except for the system Bet:NAC:W where the values were extremely lower. This shows that NADES formed mainly by sugars and some amino acids do not have main effects in the alteration of solutions' pH, which is a positive indication for the envisaged application.

NAC stands for *N*-acetyl-cysteine as seen, and it is the acetylated form of the amino acid L-cysteine. It is used as a medicine to treat paracetamol overdose and also has notable antioxidant and anti-inflammatory capabilities. [117] Despite provoking a pH of roughly 2.65, it did not cause cytotoxicity in ARPE-19 cells as shown in the 3.1.2 section. Additionally, in ocular formulations excipients to adjust the final formulation pH, such as hydrochloric acid and sodium hydroxide, thus using a system that naturally has acidic characteristics is acceptable as long as the final form is adjusted to physiological pH. The eye has some tolerability but may experience pain, inflammation, and decreased bioavailability due to increased tearing. [41] There are a lot of excipients in this situation namely HA or boric acid that are often used in many ocular formulations.

Further on this work, the systems Bet:Treh:W 4:1:10 and Bet:Treh:Gly:W 2:1:3:5 were removed from the study. These systems that combine betaine and trehalose, started to present instability even after short periods of time. Nevertheless, the system Treh:Glc:Gly:W 1:2:2:3 maintained a liquid viscous appearance and hence remained in the studies. To overcome this situation, it was added the systems Bet:Suc:W 4:1:10 and Bet:Suc:Gly:W 2:1:3:5, already presented in the previous analysis, where it was used sucrose instead of trehalose, a disaccharide with the same chemical formula ($C_{12}H_{22}O_{11}$) but with different geometrical structures.

3.2.4 Refractive Index

The action of light refraction is what makes possible the whole eye functionalities. The tear film acts as the first refractive component of the eye and changes in its dynamics might result in both visual and ocular surface discomfort. [118]
Therefore, the refractive index of NADES solutions was measured and are presented in Table 3.3

Table 3.3 - Refractive Indexes of NADES samples in PBS. Values obtained at temperatures between 18 and 24 ºC

Sample	**Refractive Index**	
Commercial Sample - Tobrex®	1.3379	
PBS	1.3350	
HPMC 0.3% PBS	1.3359	
NADES	**5% w/v PBS**	**10% w/v PBS**
Treh:Glc:Gly:W 1:2:2:3	1.3408	1.3468
Bet:EG 1:3	1.3404	1.3457
Bet:Glc:W 5:2:12	1.3410	1.3455
Fru:Glc:Suc:W 1:1:1:11	1.3405	1.3466
Glc:Pro:Gly:W 3:5:3:20	1.3407	1.3460
Bet:Suc:Gly:W 2:1:3:5	1.3459	1.3463
Bet:Gly 1:2	1.3416	1.3470
Bet:Pro:W 1:2:10	1.3397	1.3491
Bet:NAC:W 1:1:3	1.3416	1.3472
Bet:Sorb:W 1:1:3	1.3433	1.3460
Bet:Suc:W 4:1:10	1.3407	1.3455
Bet:Suc:Pro:W 5:2:2:21	1.3405	1.3460
Gly:Glc 4:1	1.3411	1.3456

The values of the refractive index did not differ significantly across the systems. Overall, these values are higher than tear films by a small margin, which is on average 1.337, [113] and quite similar to water's, 1.3326 [119]. It is visible as well that the refractive index is slightly higher when NADES concentration increases. Patel *et al.* [120] put together many results, concluding that the refractive index of the tear films' aqueous layer gradually increases from the underside of the lipid layer in the direction of the cornea, being the precorneal tear film has a refractive index of about 1.482 toping a layer where the average refractive index is about 1.337. Having NADES results in this range allows concluding that these systems would not cause blurring and discomfort when used as delivery systems.

3.2.5 Density, surface tension, and contact angle

To develop ocular drops that properly attach to the cornea, it has to be considered not only the cornea surface but also the tear film, which has to be maintained stable and consistent over time. Instillation of

a formulation can disrupt tear film over time and provoke cell damage that consequently causes conditions like DED. [37] Surface tension allows us to understand if is maintained a stable tear film and tear film break-up time and influences eye drops spreading ability and adherence. [121] The contact angle is directly connected to wettability, which is the ability of a liquid to spread over a surface. The study of the systems designed is presented in Table 3.4.

Table 3.4 – Density, surface tension, and contact angle values of NADES solutions. Data indicated as mean + SD

Sample		**Density ($g\ cm^{-3}$)**	**Surface Tension (mN/m)**	**Contact Angle (θ)**
Commercial Sample - Tobrex®		-	-	74.7 ± 5.0
HPMC 0.3% PBS		-	43.82 ± 0.04	57.7 ± 1.4
Treh:Glc:Gly:W 1:2:2:3	5% w/v PBS	1.0209	68.28 ± 0.02	106.9 ± 0.8
	10% w/v PBS	1.0348	66.87 ± 0.08	106.5 ± 2.0
Bet:EG 1:3	5% w/v PBS	1.0118	68.18 ± 0.03	105.3 ± 1.9
	10% w/v PBS	1.0172	69.43 ± 0.01	101.7 ± 0.8
Bet:Glc:W 5:2:12	5% w/v PBS	1.0150	71.57 ± 0.01	105.9 ± 1.7
	10% w/v PBS	1.0209	70.01 ± 0.08	106.9 ± 1.2
Fru:Glc:Suc:W 1:1:1:11	5% w/v PBS	1.0192	70.71 ± 0.01	104.3 ± 1.3
	10% w/v PBS	1.0328	70.68 ± 0.08	103.0 ± 3.1
Glc:Pro:Gly:W 3:5:3:20	5% w/v PBS	1.0171	63.28 ± 0.02	102.4 ± 2.2
	10% w/v PBS	1.0287	60.28 ± 0.05	100.7 ± 3.0
Bet:Gly:Suc:W	5% w/v PBS	1.0165	70.31 ± 0.04	102.2 ± 0.9
	10% w/v PBS	1.0277	68.96 ± 0.09	104.4 ± 0.1
Bet:Gly 1:2	5% w/v PBS	1.0148	66.23 ± 0.02	106.3 ± 2.0
	10% w/v PBS	1.0239	66.68 ± 0.07	107.3 ± 2.0
Bet:Pro:W 1:2:10	5% w/v PBS	1.0129	69.24 ± 0.03	109.9 ± 0.1
	10% w/v PBS	1.0197	68.13 ± 0.09	108.0 ± 3.4
Bet:NAC:W 1:1:3	5% w/v PBS	1.0148	68.51 ± 0.03	105.7 ± 3.0
	10% w/v PBS	1.0230	67.09 ± 0.05	103.0 ± 3.1
Bet:Sorb:W 1:1:3	5% w/v PBS	1.0167	68.44 ± 0.07	108.8 ± 2.0
	10% w/v PBS	1.0221	66.30 ± 0.08	103.2 ± 3.0
Bet:Suc:W 4:1:10	5% w/v PBS	1.0150	70.94 ± 0.02	103.3 ± 2.5
	10% w/v PBS	1.0246	69.10 ± 0.04	104.9 ± 1.6
Bet:Suc:Pro:W 5:2:2:21	5% w/v PBS	1.0162	66.30 ± 0.05	101.9 ± 2.3
	10% w/v PBS	1.0258	66.51 ± 0.04	108.0 ± 1.3
Gly:Glc 4:1	5% w/v PBS	1.0189	70.70 ± 0.02	103.0 ± 2.9
	10% w/v PBS	1.0316	64.17 ± 0.06	103.9 ± 1.1

Regarding surface tension, lower values lead to a fast spreading over the ocular surface without blinking. The physiological range at the air/tear fluid interface is 40-46 mN/m [121], which is considerably lower than the results obtained for the systems herein tested, which were closer to water values, ca. 72 mN/m at room temperature. [122] Surface tension above the physiological range is anticipated to result in the appearance of tear film instability which is correlated with dry eye syndrome. [121] Additionally, higher values of surface tension lead to an increase in the drop volume. With a superior drop volume, increases the amount of drug released in a single dose. Furthermore, a higher drop volume increases the washout as referred to in section 1.5 which has the opposite effect: loss of drug intake. [123] Thus, this property then must be considered to bring closer to the physiological values without compromising the other parameters. It is important not only to have a stable tear film but also to maintain the efficacy of the eye drops, which can be easily compromised as mentioned. Nevertheless, Han *et al*. [124] evaluated several ophthalmic formulations available in the market. interestingly, despite the expectation of the range to be very similar to normal tears, the reality was different, with many of them showing higher values of surface tension. For example, TheraTears® Lubricant Eye Drops presented a surface tension of 70.9 mN/m, even higher than most of our NADES solutions. According to this, our NADES solutions presented values of surface tension within values found in commercial samples and therefore do not pose a limitation for their application in ocular formulations Yet, in a future formulation, it will be possible to slightly decrease these surface tension values through the addition of a small percentage of a polymer or other substances.

In which concerns the contact angle, some experimental considerations must be made before analyzing the results. First, the fact that contact angles could only be measured by sessile drop method may lead to the possibility to have inconsistency with the *in vivo* reality. It would be ideal to use the captive bubble method, which allows the surface to be hydrated, thus more analogous to *in vivo*. Furthermore, the hardware and software are not consistent, forcing to multiple measurements and consequently errors. It is well known that wettability increases as the contact angle decreases. However, while applying this method, it is also necessary to consider the surface used. Although the aim of having good wettability is to achieve contact angles lower than 90º, this requires testing on a surface that is generally comparable to reality, which did not occur in this case. The results presented here show prominent values in the hydrophobic PTFE surface, resulting in an opposite effect. In this case, it can be concluded that the systems' formulations have poor wettability in hydrophobic surfaces, which for the goal application is a good result, it means that NADES formulations present hydrophilic properties, necessary to be applied as an ophthalmic solution.

Bock *et al.* [37] used the sessile drop method to test four commercial eye drops on three different surfaces. Hydrophobic polyethylene terephthalate, similar to PTFE, was used, as well as glass which is hydrophilic and also cell monolayers of human corneal epithelial cells. All eye drops had similar values, and the biggest change was the surfaces used to acquire these contact angle values. The contact angles on the glass surface ranged from 42.9° to 48.9° and those on the hydrophobic surface ranged from 105.7° to 112.1°, coincident with the values that were obtained in this work on a similar surface. The same authors observed that when eye drops reached the monolayer of corneal cells, they spread

throughout the surface immediately after the drop hit the layer. Consequently, it can be postulated that these NADES formulations may behave in the same form under diverse conditions.

HPMC's measurements also sustain that this polymer has better properties to suit these ophthalmic formulations and it would be necessary to determine the behavior of the combined NADES with polymers, not only to enhance viscosities but also to promote better surface tension but also to uniform dispersion of the water-insoluble particles in aqueous solutions. [125]

3.3 Antioxidant activity stability

In previous works developed by the group, three compounds with known antioxidant activity, namely rutin, taurine, and resveratrol, were dissolved in three eutectic systems studied in this work. [94], [95] Under the scope of this thesis, the stability of the antioxidants in these formulations was assessed at 1, 3, and 6 months. Here is a representative example, in Figure 3.6, with the system Bet:Treh:W the remaining graphs present in Annex A.4. The antioxidant activity in this experiment is represented as %RSA.

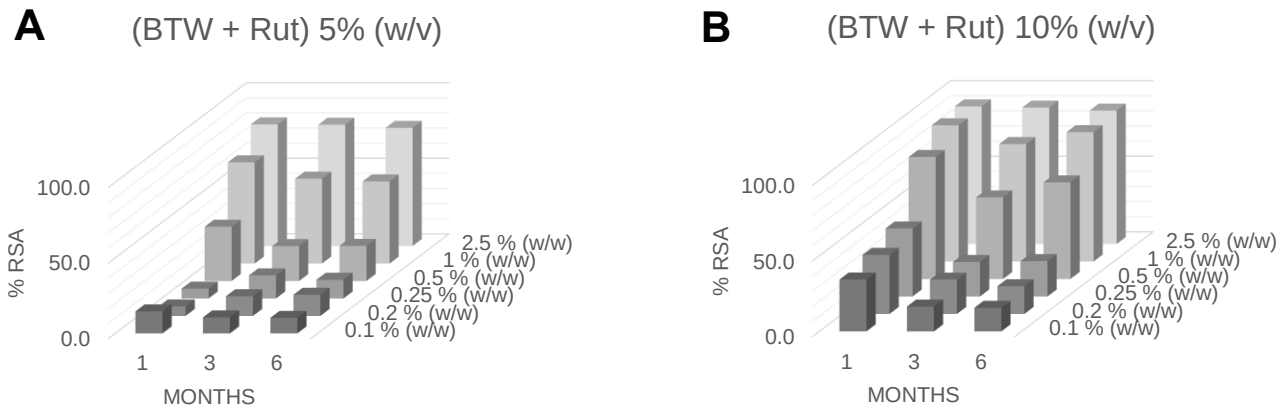

Figure 3.6 - Variation of %RSA of the system Bet:Treh:W (BTW) with rutin (Rut) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.3 to 5.5 % in graph A and 0.1 to 8.9 % in graph B.

All systems revealed a decrease in antioxidant activity after 6 months, visible in the case of NADES with rutin, where the most concentrated mixtures are the ones that have shown a lower reduction. In the case of resveratrol, the behavior was more balanced between all concentrations. In taurine, however, it is not possible to draw any conclusions due to its low and inconsistent values of %RSA, throughout the 6 months. lower than 10% in the majority, being too irregular in the measurements throughout the 6 months. It is probably near or below the limit of quantification.

In general, from the results obtained, is possible to conclude that these compounds incorporated in NADES can be used for up to 6 months which is higher than the normal expiration date of common

ocular formulations. It would be interesting to evaluate if this measurement in flasks that were never opened through this period would have the same values.

Rutin alone presents a higher %RSA than when is dissolved in the NADES mixture, however, it is important to consider that this measurement was performed in methanol and rutin has low solubility in water, hence the necessity to test this antioxidant in these systems. Nevertheless, those results need to be replicated to assess their accuracy. Additionally, as reported in the previous works the system Bet:Treh:Gly:W (BTGW) is less concentrated in rutin [95], and consequently presents the lowest antioxidant activity, being less than half compared with other systems. (Figure 3.6)

Taurine as previously mentioned has the lowest %RSA, with values lower than 20% as visible in Figure A.6-A.7. This shows that the mechanism of action of this amino-sulfonic acid is different from the rest of the antioxidants and that it does not interfere with free radical scavenging. Other *in vitro* antioxidant assays could give this information further on. [94]

The latter, resveratrol, has a similar aspect to rutin, but with lower antioxidant activity values. This result can mean two possible explanations, the compound has less antioxidant activity by being less powerful in free radical scavenging or, these results are masked, and this compound also interacts with other routes of the antioxidant defense system.

There were no performed controls of the antioxidants in an aqueous solution due to the simple fact that rutin and resveratrol are poorly soluble, and it would not be possible to achieve the same concentrations, it would precipitate.

Another consideration to be made is the interaction of the antioxidant solubilized in the NADES which consequently is diluted into an aqueous solution. The mixtures with the systems Bet:Treh:W and Bet: EG with rutin at 2.5% (w/w) when solubilized in water after a short period of time, rutin started to precipitate, thus be necessary stirring to resolubilize. The same behavior occurs with the same systems with rutin at 1% (w/w) solubilized at 10% (w/v) in water and in the same concentrations with Bet:Treh:W with resveratrol. Nevertheless, these systems can solubilize antioxidants that maintain activity for a long time and with these results, it is possible to infer that these systems with antioxidants solubilized could be used as ocular drug delivery.

Additionally, despite the presence of poorly soluble water drugs in aqueous ophthalmic formulations, dose inaccuracies are frequent, due to uneven dilution of the suspension after prolonged standing. [125] Finding a drug system that enhances the solubility of these compounds has huge benefits in terms of dosage uniformity. NADES can bring those properties acting as a third phase mimicking nature.

4. CONCLUSION

The results presented in this project suggest that NADES are potential excipients for ocular applications, especially ocular drops. NADES solutions have low cytotoxicity, especially at 5% (w/v) concentration. Then, it was observed that these presented newtonian behavior, yet at the concentrations tested, viscosity values lower than expected, presenting values that indicate that the residence time was not enhanced, but still within the range found in commercial samples of eye drops.

The fact that these solutions are hyperosmolar is a concern, however, there are already in market ocular formulations with higher osmolarities suggesting that this characteristic is not a limitation of their use.

Regarding the pH and refractive index, we have shown that these are within the optimal range. Despite presenting higher values of surface tension, we still believe that this is not a limitation, and using appropriate additives it would be possible to decrease these values to optimal ones.

Overall, all systems presented similar behaviors; however, cytotoxicity and osmolality differences can be observed between some of them, namely the systems Bet:NAC:W, Fru:Glc:Suc:W, and Bet:Gly which showed improved properties. Although the system Glc:Pro:Gly:W showed the best results in terms of cytotoxicity and osmolality, it showed less stability over time.

In summary, with these results, it was possible to show that NADES are potential candidates to be used as a drug delivery system.

5. FUTURE PERSPECTIVES

In future work, it will be necessary to assess several characteristics and perform more tests. As components to be added to human eyes, it will be necessary to determine the antimicrobial activity and its stability over time with open and closed containers and its interaction with light, evaluating light and dark preservation. Also in that category, it is necessary to perform more stability assays to evaluate possible changes in the formulation such as smell and color throughout and at different temperatures. The formulation must be tested with the rest of the excipients needed to observe its compatibility.

Furthermore, it will also be necessary to test drop size, so that the eye is not overfilled after drop instillation, which can also cause a decrease in bioavailability. Additionally, the particle size of the product needs to be evaluated, because it has a considerable impact on physical stability and ocular bioavailability, being needed to be smaller than 10 µm to avoid ocular irritation.

Moreover, ocular transparency is another characteristic to consider preventing adverse effects in patients.

As important, it is necessary to determine and evaluate the mucoadhesive properties of formulations, which will have a great impact on the adherence of the active compounds on the surface and consequently becoming systemically active.

Regarding the incorporated antioxidants is necessary to determine the *in vitro* ability to inhibit ROS production.

Lastly, other relevant compounds with antioxidant activity must be considered.

6. REFERENCES

[1] R. Bourne *et al.*, “Trends in prevalence of blindness and distance and near vision impairment over 30 years: an analysis for the Global Burden of Disease Study,” *Lancet Glob. Heal.*, vol. 9, no. 2, pp. e130–e143, Feb. 2021, doi: 10.1016/S2214-109X(20)30425-3.

[2] J. D. Steinmetz *et al.*, “Causes of blindness and vision impairment in 2020 and trends over 30 years, and prevalence of avoidable blindness in relation to VISION 2020: the Right to Sight: an analysis for the Global Burden of Disease Study,” *Lancet Glob. Heal.*, vol. 9, no. 2, pp. e144–e160, Feb. 2021, doi: 10.1016/S2214-109X(20)30489-7.

[3] U. Chakravarthy, E. Biundo, R. O. Saka, C. Fasser, R. Bourne, and J.-A. Little, “The Economic Impact of Blindness in Europe,” *Ophthalmic Epidemiol.*, vol. 24, no. 4, pp. 239–247, Jul. 2017, doi: 10.1080/09286586.2017.1281426.

[4] “Blindness and vision impairment,” *WHO*, Oct. 14, 2021. https://www.who.int/news-room/fact-sheets/detail/blindness-and-visual-impairment (accessed Jun. 02, 2022).

[5] S. Makin, “Four technologies that could transform the treatment of blindness A decade ago, clinicians had nothing to offer most people affected by retinal degeneration. Breakthroughs in genetics, bionics and stem-cell therapy are changing that,” *Nature*, vol. 568, no. 7751, 2019.

[6] K.-J. Cheng, C.-M. Hsieh, K. Nepali, and J.-P. Liou, “Ocular Disease Therapeutics: Design and Delivery of Drugs for Diseases of the Eye,” *J. Med. Chem.*, vol. 63, no. 19, pp. 10533–10593, Oct. 2020, doi: 10.1021/acs.jmedchem.9b01033.

[7] J. W. Kiel, “The Ocular Circulation,” *Colloq. Ser. Integr. Syst. Physiol. From Mol. to Funct.*, vol. 3, no. 1, pp. 1–81, 2011, doi: 10.4199/c00024ed1v01y201012isp012.

[8] E. Addo, O. A. Bamiro, and R. Siwale, “Anatomy of the eye and common diseases affecting the eye,” in *Ocular Drug Delivery: Advances, Challenges and Applications*, Springer International Publishing, 2016, pp. 11–25. doi: 10.1007/978-3-319-47691-9_2.

[9] Blausen.com staff, “Medical gallery of Blausen Medical 2014,” *WikiJournal Med.*, vol. 1, no. 2, 2014, doi: 10.15347/wjm/2014.010.

[10] K. Boyd and D. Turbert, “Eye Anatomy: Parts of the Eye and How We See,” *American Academy of Ophthalmology*, Mar. 09, 2021. https://www.aao.org/eye-health/anatomy/parts-of-eye (accessed Jun. 05, 2022).

[11] S. M. Gouveia and J. M. Tiffany, “Human tear viscosity: An interactive role for proteins and lipids,” *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1753, no. 2, pp. 155–163, 2005, doi: 10.1016/j.bbapap.2005.08.023.

[12] P. Sunkireddy, S. N. Jha, J. R. Kanwar, and S. C. Yadav, “Natural antioxidant biomolecules promises future nanomedicine based therapy for cataract,” *Colloids Surfaces B Biointerfaces*, vol. 112, pp. 554–562, Dec. 2013, doi: 10.1016/j.colsurfb.2013.07.068.

[13] S. D. Varma, D. Chand, Y. R. Sharma, J. F. Kuck, and R. D. Richards, “Oxidative stress on lens and cataract formation: role of light and oxygen,” *Curr. Eye Res.*, vol. 3, no. 1, pp. 35–58, Jan. 1984, doi: 10.3109/02713688408997186.

[14] M. A. Babizhayev and Y. E. Yegorov, “Reactive Oxygen Species and the Aging Eye: Specific Role of Metabolically Active Mitochondria in Maintaining Lens Function and in the Initiation of the Oxidation-Induced Maturity Onset Cataract-A Novel Platform of Mitochondria-Targeted Antioxidants With Broad Therapeutic Potential for Redox Regulation and Detoxification of Oxidants in Eye Diseases,” *Am. J. Ther.*, vol. 23, pp. 98–117, 2016, [Online]. Available: www.americantherapeutics.com

[15] P. A. Asbell, I. Dualan, J. Mindel, D. Brocks, M. Ahmad, and S. Epstein, “Age-related cataract,” *Lancet*, vol. 365, no. 9459, pp. 599–609, Feb. 2005, doi: 10.1016/S0140-6736(05)17911-2.
[16] M. Erdurmuş, H. Simavlı, and B. Aydın, “Cataracts: An Overview,” in *Handbook of Nutrition, Diet, and the Eye*, Elsevier, 2019, pp. 231–244. doi: 10.1016/b978-0-12-815245-4.00014-4.
[17] S. Bungau *et al.*, “Health Benefits of Polyphenols and Carotenoids in Age-Related Eye Diseases,” *Oxidative Medicine and Cellular Longevity*, vol. 2019. Hindawi Limited, 2019. doi: 10.1155/2019/9783429.
[18] R. N. Weinreb, T. Aung, and F. A. Medeiros, “The pathophysiology and treatment of glaucoma: A review,” *JAMA - Journal of the American Medical Association*, vol. 311, no. 18. American Medical Association, pp. 1901–1911, May 14, 2014. doi: 10.1001/jama.2014.3192.
[19] M. C. Moreno, J. Campanelli, P. Sande, D. A. Sáenz, M. I. Keller Sarmiento, and R. E. Rosenstein, “Retinal Oxidative Stress Induced by High Intraocular Pressure,” *Free Radic. Biol. Med.*, vol. 37, no. 6, pp. 803–812, Sep. 2004, doi: 10.1016/j.freeradbiomed.2004.06.001.
[20] V. B. Voleti and J. P. Hubschman, “Age-related eye disease,” *Maturitas*, vol. 75, no. 1. Elsevier Ireland Ltd, pp. 29–33, 2013. doi: 10.1016/j.maturitas.2013.01.018.
[21] V. Chrysostomou, F. Rezania, I. A. Trounce, and J. G. Crowston, “Oxidative stress and mitochondrial dysfunction in glaucoma,” *Curr. Opin. Pharmacol.*, vol. 13, no. 1, pp. 12–15, Feb. 2013, doi: 10.1016/j.coph.2012.09.008.
[22] E. Lefevere, A. K. Toft-Kehler, R. Vohra, M. Kolko, L. Moons, and I. Van Hove, “Mitochondrial dysfunction underlying outer retinal diseases,” *Mitochondrion*, vol. 36, pp. 66–76, Sep. 2017, doi: 10.1016/j.mito.2017.03.006.
[23] A. Gorusupudi, K. Nelson, and P. S. Bernstein, “The age-related eye disease 2 study: Micronutrients in the treatment of macular degeneration,” *Adv. Nutr.*, vol. 8, no. 1, pp. 40–53, 2017, doi: 10.3945/an.116.013177.
[24] A. G. Layana *et al.*, “Vitamin D and age-related macular degeneration,” *Nutrients*, vol. 9, no. 10, pp. 1–15, 2017, doi: 10.3390/nu9101120.
[25] W. Du, Y. An, X. He, D. Zhang, and W. He, “Protection of kaempferol on oxidative stress-induced retinal pigment epithelial cell damage,” *Oxid. Med. Cell. Longev.*, vol. 2018, 2018, doi: 10.1155/2018/1610751.
[26] R. A. Kowluru and P. S. Chan, “Oxidative stress and diabetic retinopathy,” *Exp. Diabesity Res.*, vol. 2007, 2007, doi: 10.1155/2007/43603.
[27] J. T. Eells, “Mitochondrial dysfunction in the aging retina,” *Biology*, vol. 8, no. 2. MDPI AG, Jun. 01, 2019. doi: 10.3390/biology8020031.
[28] “The Definition and Classification of Dry Eye Disease: Report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007),” *Ocul. Surf.*, vol. 5, no. 2, pp. 75–92, Apr. 2007, doi: 10.1016/S1542-0124(12)70081-2.
[29] P. Agarwal, J. P. Craig, and I. D. Rupenthal, “Formulation considerations for the management of dry eye disease,” *Pharmaceutics*, vol. 13, no. 2, pp. 1–19, 2021, doi: 10.3390/pharmaceutics13020207.
[30] V. Valim, V. F. M. Trevisani, J. M. de Sousa, V. S. Vilela, and R. Belfort, “Current Approach to Dry Eye Disease,” *Clin. Rev. Allergy Immunol.*, vol. 49, no. 3, pp. 288–297, 2015, doi: 10.1007/s12016-014-8438-7.
[31] J. Kruk, K. Kubasik-Kladna, and H. Y. Aboul-Enein, “The Role Oxidative Stress in the Pathogenesis of Eye Diseases: Currnt Status and a Dual Role of Physical Activity,” *Mini-Reviews Med. Chem.*, vol. 16, no. 3, pp. 241–257, Nov. 2015, doi: 10.2174/1389557516666151120114605.
[32] S. Seen and L. Tong, “Dry eye disease and oxidative stress,” *Acta Ophthalmol.*, vol. 96, no. 4, pp. e412–e420, Jun. 2018, doi: 10.1111/aos.13526.
[33] N. Jaenen, C. Baudouin, P. Pouliquen, G. Manni, A. Figueiredo, and T. Zeyen, “Ocular Symptoms and Signs with Preserved and Preservative-Free Glaucoma Medications,” *Eur. J. Ophthalmol.*, vol. 17, no. 3, pp. 341–349, May 2007, doi: 10.1177/112067210701700311.
[34] A. Patel, “Ocular drug delivery systems: An overview,” *World J. Pharmacol.*, vol. 2, no. 2, p. 47, 2013, doi: 10.5497/wjp.v2.i2.47.
[35] V. Gote, S. Sikder, J. Sicotte, and D. Pal, “Ocular drug delivery: Present innovations and future challenges,” *Journal of Pharmacology and Experimental Therapeutics*, vol. 370, no. 3. American Society for Pharmacology and Experimental Therapy, pp. 602–624, 2019. doi: 10.1124/jpet.119.256933.
[36] D. Achouri, K. Alhanout, P. Piccerelle, and V. Andrieu, “Recent advances in ocular drug delivery,” *Drug Dev. Ind. Pharm.*, vol. 39, no. 11, pp. 1599–1617, 2013, doi: 10.3109/03639045.2012.736515.

[37] U. Bock, V. Deylen D, J. M, D. M, S. C, and R. S, “Development of In Vitro Methodologies to Investigate Binding by Sodium Hyaluronate in Eye Drops to Corneal Surfaces,” *Open Ophthalmol. J.*, vol. 12, no. 1, pp. 226–240, Jul. 2018, doi: 10.2174/1874364101812010226.
[38] L. Bourlais, L. Acar, H. Zia, P. A. Sado, T. Needham, and R. Leverg, “Ophthalmic Drug Delivery Systems Recent Advances,” vol. 17, no. 1, pp. 33–58, 1998.
[39] J. kour, N. Kumari, and B. Sapra, “Ocular prodrugs: Attributes and challenges,” *Asian J. Pharm. Sci.*, vol. 16, no. 2, pp. 175–191, 2021, doi: 10.1016/j.ajps.2020.08.002.
[40] K. Cholkar, S. P. Patel, A. D. Vadlapudi, and A. K. Mitra, “Novel strategies for anterior segment ocular drug delivery,” *J. Ocul. Pharmacol. Ther.*, vol. 29, no. 2, pp. 106–123, 2013, doi: 10.1089/jop.2012.0200.
[41] P. Baranowski, B. Karolewicz, M. Gajda, and J. Pluta, “Ophthalmic drug dosage forms: Characterisation and research methods,” *Sci. World J.*, vol. 2014, 2014, doi: 10.1155/2014/861904.
[42] H. Abdelkader and R. G. Alany, “Controlled and Continuous Release Ocular Drug Delivery Systems : Pros and Cons,” pp. 421–430, 2012.
[43] H. Zhu and A. Chauhan, “Effect of Viscosity on Tear Drainage and Ocular Residence Time,” vol. 85, no. 8, pp. 715–725, 2008.
[44] M. Q. Rahman, K. Chuah, E. C. A. Macdonald, J. P. M. Trusler, and K. Ramaesh, “The effect of pH , dilution , and temperature on the viscosity of ocular lubricants — shift in rheological parameters and potential clinical significance,” *Eye*, vol. 26, no. 12, pp. 1579–1584, 2012, doi: 10.1038/eye.2012.211.
[45] I. Zaki, P. Fitzgerald, and J. G. Hardy, “A comparison of the effect of viscosity on the precorneal residence of solutions in rabbit and man,” pp. 463–466, 1986.
[46] I. Krämer, M. Haber, and A. Duis, “Formulation Requirements for the Ophthalmic Use of Antiseptics”.
[47] U. Stahl, M. Willcox, and F. Stapleton, “Osmolality and tear film dynamics,” *Clinical and Experimental Optometry*, vol. 95, no. 1. pp. 3–11, 2012. doi: 10.1111/j.1444-0938.2011.00634.x.
[48] D. L. Williams, “Oxidative Stress and the Eye,” *Veterinary Clinics of North America - Small Animal Practice*, vol. 38, no. 1. pp. 179–192, Jan. 2008. doi: 10.1016/j.cvsm.2007.10.006.
[49] Y.-J. Hsueh, Y.-N. Chen, Y.-T. Tsao, C.-M. Cheng, W.-C. Wu, and H.-C. Chen, “The Pathomechanism, Antioxidant Biomarkers, and Treatment of Oxidative Stress-Related Eye Diseases,” *Int. J. Mol. Sci.*, vol. 23, no. 3, p. 1255, Jan. 2022, doi: 10.3390/ijms23031255.
[50] H. Sies, C. Berndt, and D. P. Jones, “Oxidative Stress,” *Annu. Rev. Biochem.*, vol. 86, no. 1, pp. 715–748, Jun. 2017, doi: 10.1146/annurev-biochem-061516-045037.
[51] R. Sotler *et al.*, “PROOXIDANT ACTIVITIES OF ANTIOXIDANTS AND THEIR IMPACT ON HEALTH,” *Acta clinica Croatica*, vol. 58, no. 4. NLM (Medline), pp. 726–736, Dec. 01, 2019. doi: 10.20471/acc.2019.58.04.20.
[52] M. Nita and A. Grzybowski, “The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults,” *Oxidative Medicine and Cellular Longevity*, vol. 2016. Hindawi Limited, 2016. doi: 10.1155/2016/3164734.
[53] B. R. Hammond, B. A. Johnson, and E. R. George, “Oxidative photodegradation of ocular tissues: Beneficial effects of filtering and exogenous antioxidants,” *Experimental Eye Research*, vol. 129. Academic Press, pp. 135–150, Dec. 01, 2014. doi: 10.1016/j.exer.2014.09.005.
[54] L. Adler, N. P. Boyer, C. Chen, Z. Ablonczy, R. K. Crouch, and Y. Koutalos, “The 11-cis Retinal Origins of Lipofuscin in the Retina,” in *Progress in Molecular Biology and Translational Science*, 2015, vol. 134, pp. e1–e12. doi: 10.1016/bs.pmbts.2015.07.022.
[55] M. Rhone and A. Basu, “Phytochemicals and age-related eye diseases,” *Nutr. Rev.*, vol. 66, no. 8, pp. 465–472, 2008, doi: 10.1111/j.1753-4887.2008.00078.x.
[56] K. O. Chu and C. P. Pang, “Herbal molecules in eye diseases,” *Taiwan J. Ophthalmol.*, vol. 4, no. 3, pp. 103–109, 2014, doi: 10.1016/j.tjo.2014.03.005.
[57] Z. Xu, T. Sun, W. Li, and X. Sun, “Inhibiting effects of dietary polyphenols on chronic eye diseases,” *J. Funct. Foods*, vol. 39, no. October, pp. 186–197, 2017, doi: 10.1016/j.jff.2017.10.031.
[58] D. Ghosh and T. Konishi, “Anthocyanins And Anthocyanin-Rich Extracts,” *Asia Pac J Clin Nutr*, vol. 16, no. 2, pp. 200–208, 2007.
[59] C. Bola, H. Bartlett, and F. Eperjesi, “Resveratrol and the eye: Activity and molecular mechanisms,” *Graefe’s Arch. Clin. Exp. Ophthalmol.*, vol. 252, no. 5, pp. 699–713, 2014, doi: 10.1007/s00417-014-2604-8.
[60] C. Luna *et al.*, “Resveratrol prevents the expression of glaucoma markers induced by chronic

oxidative stress in trabecular meshwork cells," *Food Chem. Toxicol.*, vol. 47, no. 1, pp. 198–204, 2009, doi: 10.1016/j.fct.2008.10.029.
[61] Y. Li, L. Qin, L. Ying, H. Dong, and D. Wang, "Rutin prevents retinal ganglion cell death and exerts protective effects by regulating transforming growth factor-β2/smad2/3akt/PTEN signaling in experimental rat glaucoma," *Trop. J. Pharm. Res.*, vol. 18, no. 5, pp. 985–993, 2019, doi: 10.4314/tjpr.v18i5.11.
[62] M. Isai, M. Sakthivel, E. Ramesh, P. A. Thomas, and P. Geraldine, "Prevention of selenite-induced cataractogenesis by rutin in Wistar rats.," *Mol. Vis.*, vol. 15, no. November, pp. 2570–2577, 2009.
[63] D. Kelly *et al.*, "Cognitive function and its relationship with macular pigment optical density and serum concentrations of its constituent carotenoids," *J. Alzheimer's Dis.*, vol. 48, no. 1, pp. 261–277, 2015, doi: 10.3233/JAD-150199.
[64] J. Widomska, "Why has Nature Chosen Lutein and Zeaxanthin to Protect the Retina?," *J. Clin. Exp. Ophthalmol.*, vol. 05, no. 01, pp. 1–22, 2014, doi: 10.4172/2155-9570.1000326.
[65] S. Y. Sarialtin and T. Coban, "An overview on the role of macular Xanthophylls in ocular diseases," *Rec. Nat. Prod.*, vol. 12, no. 2, pp. 107–120, 2018, doi: 10.25135/RNP.14.17.07.067.
[66] R. A. Bone, J. T. Landrum, Y. Cao, A. N. Howard, and F. Alvarez-Calderon, "Macular pigment response to a supplement containing meso-zeaxanthin, lutein and zeaxanthin.," *Nutr. Metab. (Lond).*, vol. 4, p. 12, May 2007, doi: 10.1186/1743-7075-4-12.
[67] S. P. Sundelin and S. E. G. Nilsson, "Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants," *Free Radic. Biol. Med.*, vol. 31, no. 2, pp. 217–225, 2001, doi: 10.1016/S0891-5849(01)00573-1.
[68] A. A. Heinämäki, A. S. H. Muhonen, and R. S. Piha, "Taurine and other free amino acids in the retina, vitreous, lens, irisciliary body, and cornea of the rat eye," *Neurochem. Res.*, vol. 11, no. 4, pp. 535–542, 1986, doi: 10.1007/BF00965323.
[69] C. J. Jong, J. Azuma, and S. Schaffer, "Mechanism underlying the antioxidant activity of taurine: Prevention of mitochondrial oxidant production," *Amino Acids*, vol. 42, no. 6, pp. 2223–2232, 2012, doi: 10.1007/s00726-011-0962-7.
[70] D. Rusciano, A. M. Roszkowska, C. Gagliano, and S. Pezzino, "Free amino acids: an innovative treatment for ocular surface disease," *Eur. J. Pharmacol.*, vol. 787, pp. 9–19, 2016, doi: 10.1016/j.ejphar.2016.04.029.
[71] K. Robinson, C. Mock, and D. Liang, "Pre-formulation studies of resveratrol.," *Drug Dev. Ind. Pharm.*, vol. 41, no. 9, pp. 1464–1469, 2015, doi: 10.3109/03639045.2014.958753.
[72] T. I. Khalifa, F. J. Muhtadi, and M. M. A. Hassan, "Rutin," vol. 12, K. B. T.-A. P. of D. S. Florey, Ed. Academic Press, 1983, pp. 623–681. doi: https://doi.org/10.1016/S0099-5428(08)60177-X.
[73] A. Paiva, R. Craveiro, I. Aroso, M. Martins, R. L. Reis, and A. R. C. Duarte, "Natural Deep Eutectic Solvents – Solvents for the 21st Century," *ACS Sustain. Chem. Eng.*, vol. 2, no. 5, pp. 1063–1071, May 2014, doi: 10.1021/sc500096j.
[74] D. A. Alonso, A. Baeza, R. Chinchilla, G. Guillena, I. M. Pastor, and D. J. Ramón, "Deep Eutectic Solvents: The Organic Reaction Medium of the Century," *European J. Org. Chem.*, vol. 2016, no. 4, pp. 612–632, Feb. 2016, doi: https://doi.org/10.1002/ejoc.201501197.
[75] A. P. Abbott, G. Capper, D. L. Davies, R. K. Rasheed, and V. Tambyrajah, "Novel solvent properties of choline chloride/urea mixturesElectronic supplementary information (ESI) available: spectroscopic data. See http://www.rsc.org/suppdata/cc/b2/b210714g/," *Chem. Commun.*, no. 1, pp. 70–71, Dec. 2003, doi: 10.1039/b210714g.
[76] R. Craveiro *et al.*, "Properties and thermal behavior of natural deep eutectic solvents," *J. Mol. Liq.*, vol. 215, pp. 534–540, 2016, doi: 10.1016/j.molliq.2016.01.038.
[77] B. B. Hansen *et al.*, "Deep Eutectic Solvents : A Review of Fundamentals and Applications," 2021, doi: 10.1021/acs.chemrev.0c00385.
[78] T. El Achkar, S. Fourmentin, and H. Greige-Gerges, "Deep eutectic solvents: An overview on their interactions with water and biochemical compounds," *J. Mol. Liq.*, vol. 288, p. 111028, 2019, doi: 10.1016/j.molliq.2019.111028.
[79] M. Hayyan, M. Ali, M. A. Al-saadi, A. Hayyan, I. M. Alnashef, and M. E. S. Mirghani, "Chemosphere Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents," *Chemosphere*, vol. 93, no. 2, pp. 455–459, 2013, doi: 10.1016/j.chemosphere.2013.05.013.
[80] X. Hou, Q. Liu, T. J. Smith, N. Li, and M. Zong, "Evaluation of Toxicity and Biodegradability of Cholinium Amino Acids Ionic Liquids," vol. 8, no. 3, 2013, doi: 10.1371/journal.pone.0059145.
[81] A. P. Abbott, D. Boothby, G. Capper, D. L. Davies, and R. K. Rasheed, "Deep Eutectic Solvents formed between choline chloride and carboxylic acids: Versatile alternatives to ionic liquids," *J.*

Am. Chem. Soc., vol. 126, no. 29, pp. 9142–9147, 2004, doi: 10.1021/ja048266j.
[82] Y. H. Choi *et al.*, “Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology?,” *Plant Physiol.*, vol. 156, no. 4, pp. 1701–1705, 2011, doi: 10.1104/pp.111.178426.
[83] Y. Dai, J. van Spronsen, G. J. Witkamp, R. Verpoorte, and Y. H. Choi, “Natural deep eutectic solvents as new potential media for green technology,” *Anal. Chim. Acta*, vol. 766, pp. 61–68, 2013, doi: 10.1016/j.aca.2012.12.019.
[84] A. Gertrudes, R. Craveiro, Z. Eltayari, R. L. Reis, A. Paiva, and A. R. C. Duarte, “How Do Animals Survive Extreme Temperature Amplitudes? the Role of Natural Deep Eutectic Solvents,” *ACS Sustain. Chem. Eng.*, vol. 5, no. 11, pp. 9542–9553, 2017, doi: 10.1021/acssuschemeng.7b01707.
[85] Y. Dai, G.-J. Witkamp, R. Verpoorte, and Y. H. Choi, “Tailoring properties of natural deep eutectic solvents with water to facilitate their applications,” *Food Chem.*, vol. 187, pp. 14–19, 2015, doi: https://doi.org/10.1016/j.foodchem.2015.03.123.
[86] H. Monteiro, A. Paiva, A. R. C. Duarte, and N. Galamba, “Structure and Dynamic Properties of a Glycerol-Betaine Deep Eutectic Solvent: When Does a des Become an Aqueous Solution?,” *ACS Sustain. Chem. Eng.*, vol. 10, no. 11, pp. 3501–3512, 2022, doi: 10.1021/acssuschemeng.1c07461.
[87] Y. Dai, G. J. Witkamp, R. Verpoorte, and Y. H. Choi, “Natural deep eutectic solvents as a new extraction media for phenolic metabolites in carthamus tinctorius L.,” *Anal. Chem.*, vol. 85, no. 13, pp. 6272–6278, 2013, doi: 10.1021/ac400432p.
[88] M. Faggian, S. Sut, B. Perissutti, V. Baldan, I. Grabnar, and S. Dall’Acqua, “Natural Deep Eutectic Solvents (NADES) as a tool for bioavailability improvement: Pharmacokinetics of rutin dissolved in proline/glycine after oral administration in rats: Possible application in nutraceuticals,” *Molecules*, vol. 21, no. 11, pp. 1–11, 2016, doi: 10.3390/molecules21111531.
[89] M. H. Zainal-Abidin, M. Hayyan, G. C. Ngoh, W. F. Wong, and C. Y. Looi, “Emerging frontiers of deep eutectic solvents in drug discovery and drug delivery systems,” *J. Control. Release*, vol. 316, no. September, pp. 168–195, 2019, doi: 10.1016/j.jconrel.2019.09.019.
[90] European Commission, “Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products,” *Off. J. Eur. Union*, no. 1223, pp. 342–359, 2009, doi: 10.3000/17252555.L_2009.342.eng.
[91] L. L. . Yuen, Melissa V.; Gianturco, Stephanie L.; Pavlech and A. N. Storm, Kathena D.; Yoon, SeJeong; Mattingly, “Choline Chloride: Summary Report,” 2019, Accessed: Sep. 12, 2022. [Online]. Available: https://archive.hshsl.umaryland.edu/bitstream/handle/10713/12088/Choline chloride_Final_2019_12.pdf?sequence=6&isAllowed=y
[92] L. Meneses, F. Santos, A. R. Gameiro, A. Paiva, and A. R. C. Duarte, “Preparation of Binary and Ternary Deep Eutectic Systems,” *J. Vis. Exp.*, no. 152, Oct. 2019, doi: 10.3791/60326.
[93] V. Kuete, O. Karaosmanoğlu, and H. Sivas, “Chapter 10 - Anticancer Activities of African Medicinal Spices and Vegetables,” V. B. T.-M. S. and V. from A. Kuete, Ed. Academic Press, 2017, pp. 271–297. doi: https://doi.org/10.1016/B978-0-12-809286-6.00010-8.
[94] E. V. Suyarko, “Sistemas Eutécticos Naturais com propriedades antioxidantes para o tratamento de doenças oculares.,” NOVA School of Science and Technology, 2021.
[95] S. M. E. Cabral, “Sistemas Eutécticos Naturais antioxidantes aplicados a doenças oculares,” NOVA School of Science and Technology, 2021.
[96] E. Mfotie Njoya, “Chapter 31 - Medicinal plants, antioxidant potential, and cancer,” V. R. Preedy and V. B. B. T.-C. (Second E. Patel, Eds. San Diego: Academic Press, 2021, pp. 349–357. doi: https://doi.org/10.1016/B978-0-12-819547-5.00031-6.
[97] “Determination of the activity of an antioxidant by the DPPH° assay,” *CHIMACTIV*. http://chimactiv.agroparistech.fr/en/aliments/antioxydant-dpph/principe (accessed Aug. 24, 2022).
[98] A. R. Jesus, L. Meneses, A. R. C. Duarte, and A. Paiva, “Natural deep eutectic systems, an emerging class of cryoprotectant agents,” *Cryobiology*, vol. 101, pp. 95–104, 2021, doi: 10.1016/j.cryobiol.2021.05.002.
[99] S. C. Owen, J. W. Kuo, and G. D. Prestwich, “2.14 Hyaluronic acid,” *Compr. Biomater. II*, vol. 2, no. November 2016, pp. 306–331, 2017, doi: 10.1016/B978-0-12-803581-8.10188-2.
[100] J. M. Tiffany, “The viscosity of human tears,” *Int. Ophthalmol.*, vol. 15, no. 6, pp. 371–376, 1991, doi: 10.1007/BF00137947.
[101] “Not a dry eye in the house: Eye drop rheology profiling,” *Centre for Industrial Rheology*. https://www.rheologylab.com/articles/pharma/rheology-lubricating-eye-drops/ (accessed Jul. 25, 2022).

[102] I. M. Aroso *et al.*, “Fundamental studies on natural deep eutectic solvents: physico-chemical, thermal and rheological properties,” *NWBC 2015 - 6th Nord. Wood Biorefinery Conf.*, pp. 155–160, 2015.
[103] I. M. Aroso, A. Paiva, R. L. Reis, and A. R. C. Duarte, “Natural deep eutectic solvents from choline chloride and betaine – Physicochemical properties,” *J. Mol. Liq.*, vol. 241, pp. 654–661, 2017, doi: 10.1016/j.molliq.2017.06.051.
[104] T. Altamash *et al.*, “Gas Solubility and Rheological Behavior of Natural Deep Eutectic Solvents (NADES) via Combined Experimental and Molecular Simulation Techniques,” *ChemistrySelect*, vol. 2, no. 24, pp. 7278–7295, 2017, doi: 10.1002/slct.201701223.
[105] T. Altamash *et al.*, “Gas solubility and rheological behavior study of betaine and alanine based natural deep eutectic solvents (NADES),” *J. Mol. Liq.*, vol. 256, pp. 286–295, 2018, doi: 10.1016/j.molliq.2018.02.049.
[106] R. H. Ewoldt, M. T. Johnston, and L. M. Caretta, *Experimental Challenges of Shear Rheology: How to Avoid Bad Data*. 2015. doi: 10.1007/978-1-4939-2065-5_6.
[107] M. T. Johnston and R. H. Ewoldt, “Precision rheometry: Surface tension effects on low-torque measurements in rotational rheometers,” *J. Rheol. (N. Y. N. Y).*, vol. 57, no. 6, pp. 1515–1532, 2013, doi: 10.1122/1.4819914.
[108] F. A. Che Arif, M. R. Hilmi, K. Mohd Kamal, and M. H. Ithnin, “Evaluation of 18 artificial tears based on viscosity and pH,” *Malaysian J. Ophthalmol.*, vol. 2, no. 2, pp. 96–111, 2020, doi: 10.35119/myjo.v2i2.109.
[109] W. Kapadia *et al.*, “Shear-Thinning and Temperature-Dependent Viscosity Relationships of Contemporary Ocular Lubricants,” *Transl. Vis. Sci. Technol.*, vol. 11, no. 3, pp. 1–10, 2022, doi: 10.1167/tvst.11.3.1.
[110] S. Arshinoff, I. Hofmann, and H. Nae, “Rheological behavior of commercial artificial tear solutions,” *J. Cataract Refract. Surg.*, vol. 47, no. 5, pp. 649–654, 2021, doi: 10.1097/j.jcrs.0000000000000507.
[111] J. Laihia and K. Kaarniranta, “Trehalose for Ocular Surface Health,” *Biomolecules*, vol. 10, no. 5, 2020, doi: 10.3390/biom10050809.
[112] A. Tomlinson and S. Khanal, “Assessment of tear film dynamics: Quantification approach,” *Ocul. Surf.*, vol. 3, no. 2, pp. 81–95, 2005, doi: 10.1016/S1542-0124(12)70157-X.
[113] J. P. Craig, P. A. Simmons, S. Patel, and A. Tomlinson, “Refractive index and osmolality of human tears.,” *Optom. Vis. Sci. Off. Publ. Am. Acad. Optom.*, vol. 72, no. 10, pp. 718–724, Oct. 1995, doi: 10.1097/00006324-199510000-00004.
[114] R. M. Dutescu, C. Panfil, and N. Schrage, “Osmolarity of Prevalent Eye Drops, Side Effects, and Therapeutic Approaches,” *Cornea*, vol. 34, no. 5, pp. 560–566, May 2015, doi: 10.1097/ICO.0000000000000368.
[115] F. H. Fischer and M. Wiederholt, “Human precorneal tear film pH measured by microelectrodes,” *Graefe’s Arch. Clin. Exp. Ophthalmol.*, vol. 218, no. 3, pp. 168–170, 1982, doi: 10.1007/BF02215658.
[116] M. Garcia-Valldecabres, A. López-Alemany, and M. F. Refojo, “pH Stability of ophthalmic solutions,” *Optometry*, vol. 75, no. 3, pp. 161–168, 2004, doi: 10.1016/S1529-1839(04)70035-4.
[117] M. C. D. S. Tenório, N. G. Graciliano, F. A. Moura, A. C. M. de Oliveira, and M. O. F. Goulart, “N-Acetylcysteine (NAC): Impacts on Human Health.,” *Antioxidants (Basel, Switzerland)*, vol. 10, no. 6, Jun. 2021, doi: 10.3390/antiox10060967.
[118] S. Koh, C. I. Tung, Y. Inoue, and V. Jhanji, “Effects of tear film dynamics on quality of vision,” *Br. J. Ophthalmol.*, vol. 102, no. 12, pp. 1615–1620, 2018, doi: 10.1136/bjophthalmol-2018-312333.
[119] B. K. Pierscionek, “Refractive index of the human lens surface measured with an optic fibre sensor.,” *Ophthalmic Res.*, vol. 26, no. 1, pp. 32–35, 1994, doi: 10.1159/000267371.
[120] S. Patel and L. Tutchenko, “The refractive index of the human cornea: A review,” *Contact Lens Anterior Eye*, vol. 42, no. 5, pp. 575–580, 2019, doi: 10.1016/j.clae.2019.04.018.
[121] M. Hotujac Grgurević, M. Juretić, A. Hafner, J. Lovrić, and I. Pepić, “Tear fluid-eye drops compatibility assessment using surface tension,” *Drug Dev. Ind. Pharm.*, vol. 43, no. 2, pp. 275–282, 2017, doi: 10.1080/03639045.2016.1238924.
[122] G. Vazquez, E. Alvarez, and J. M. Navaza, “Surface Tension of Alcohol Water + Water from 20 to 50 .degree.C,” *J. Chem. Eng. Data*, vol. 40, no. 3, pp. 611–614, May 1995, doi: 10.1021/je00019a016.
[123] L. Van Santvliet, K. Caljon, L. Pieters, and A. Ludwig, “Physicochemical properties, NMR spectroscopy and tolerance of inclusion complexes of antazoline and tetracaine with hydroxypropyl-β-cyclodextrin,” *Int. J. Pharm.*, vol. 171, no. 2, pp. 147–156, 1998, doi: https://doi.org/10.1016/S0378-5173(98)00171-9.

[124] K. Han, O. E. Woghiren, and R. Priefer, “Surface tension examination of various liquid oral, nasal, and ophthalmic dosage forms,” *Chem. Cent. J.*, vol. 10, no. 1, pp. 1–5, 2016, doi: 10.1186/s13065-016-0176-x.
[125] S. I. Yasueda, K. Inada, K. Matsuhisa, H. Terayama, and A. Ohtori, “Evaluation of ophthalmic suspensions using surface tension,” *Eur. J. Pharm. Biopharm.*, vol. 57, no. 2, pp. 377–382, 2004, doi: 10.1016/S0939-6411(03)00159-0.

A
APPENDIX

A.1 Viscosity as a function of shear rate

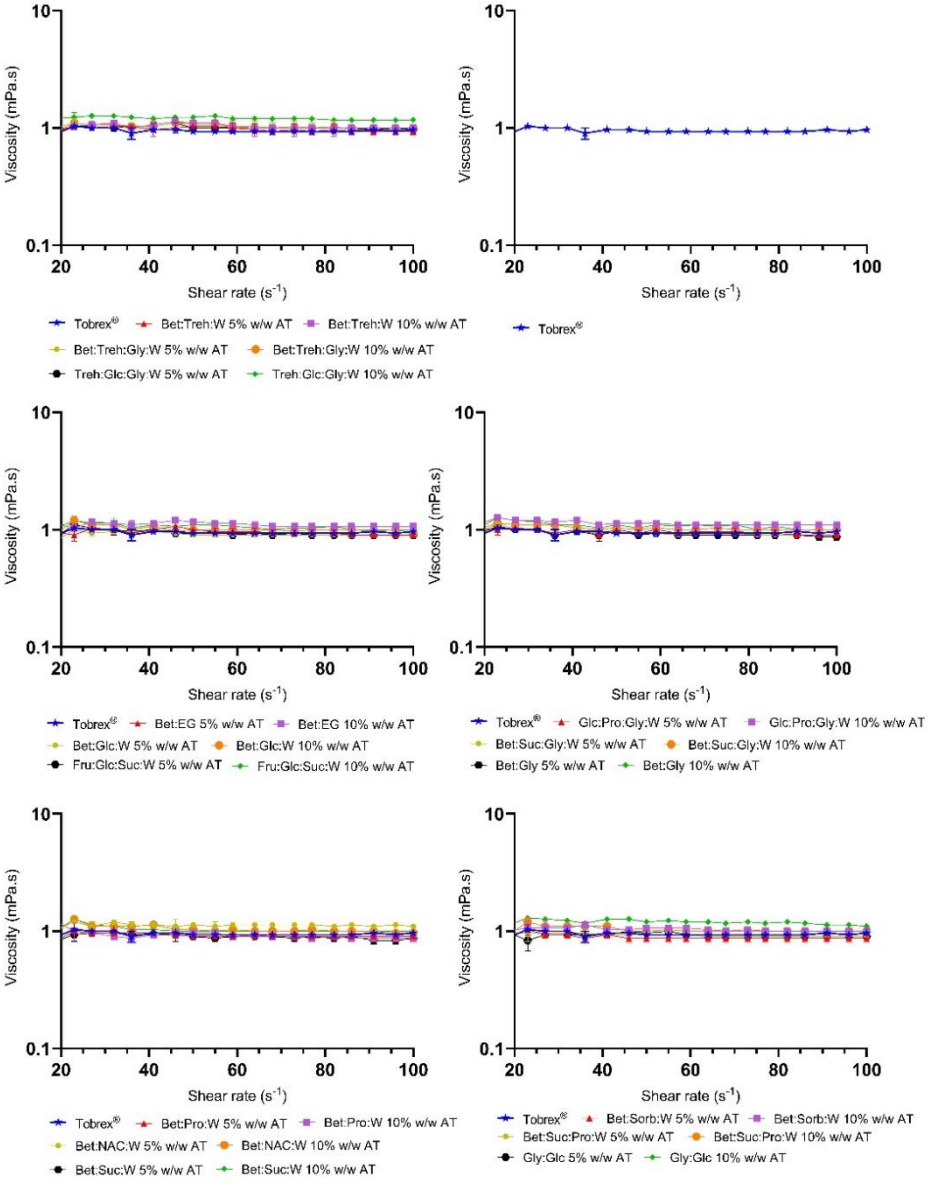

Figure A.1 - Viscosity as function of shear rate of NADES solutions and Tobrex®

A.2 Viscosity as a function of temperature

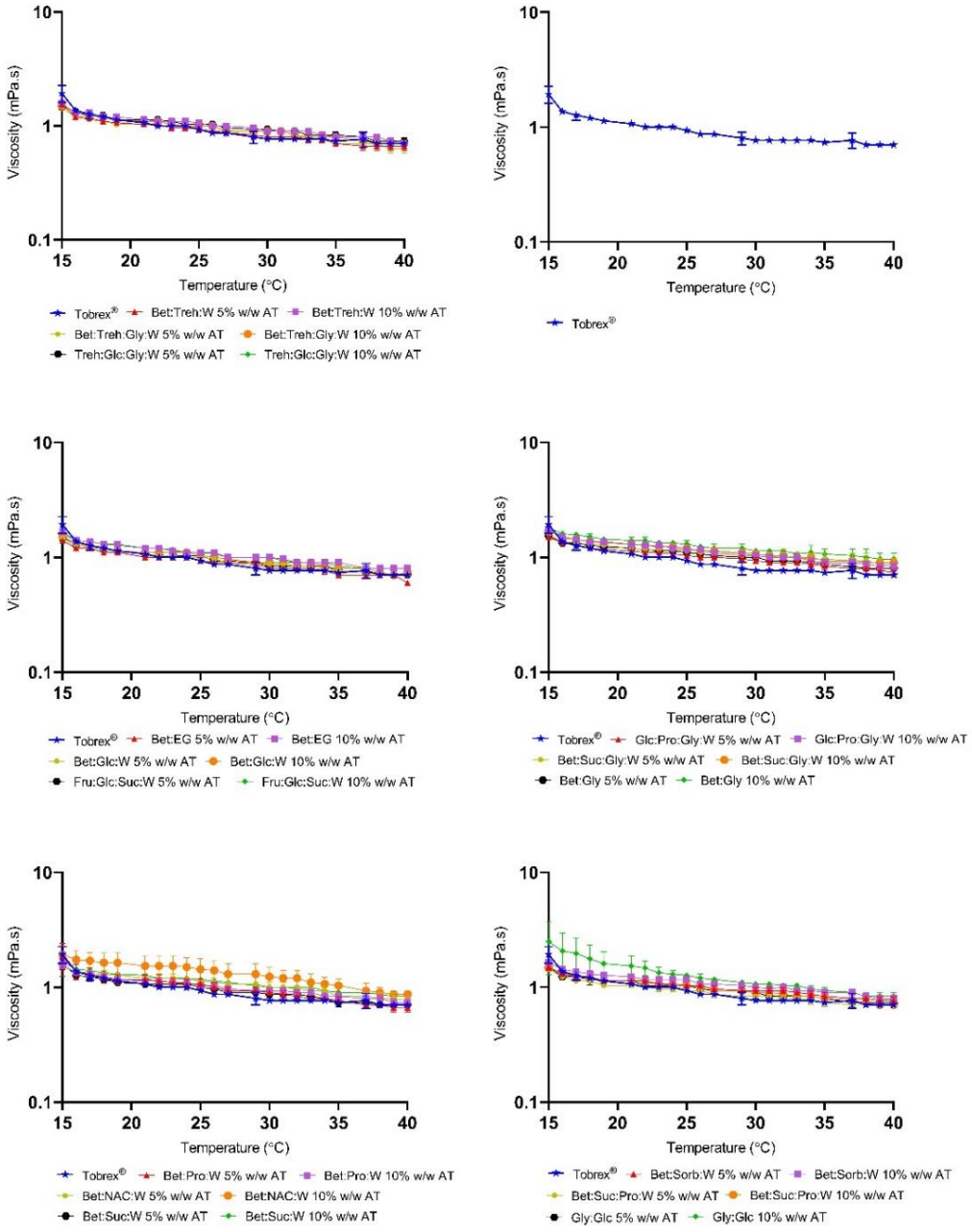

Figure A.2 - Viscosity as function of temperature of NADES solutions and Tobrex®

A.3 Viscosity at different NADES concentration

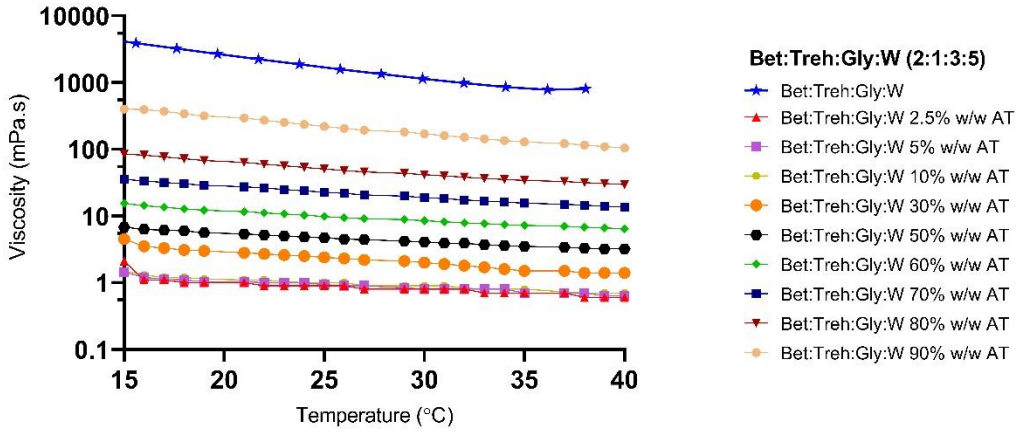

Figure A.3 - Viscosity values of the system Bet:Treh:Gly:W pure and with several AT dilutions

A.4 Antioxidant activity of NADES with natural antioxidants solubilized

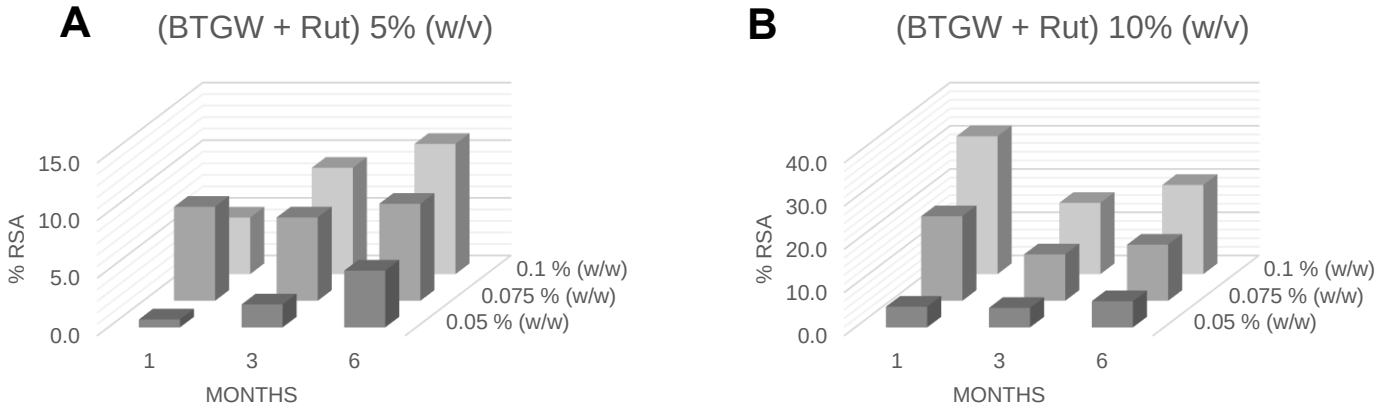

Figure A.4 - Variation of %RSA of the system Bet:Treh:Gly:W (BTGW) with rutin (Rut) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.4 to 3.6 % in graph A and 0.3 to 5.5 % in graph B.

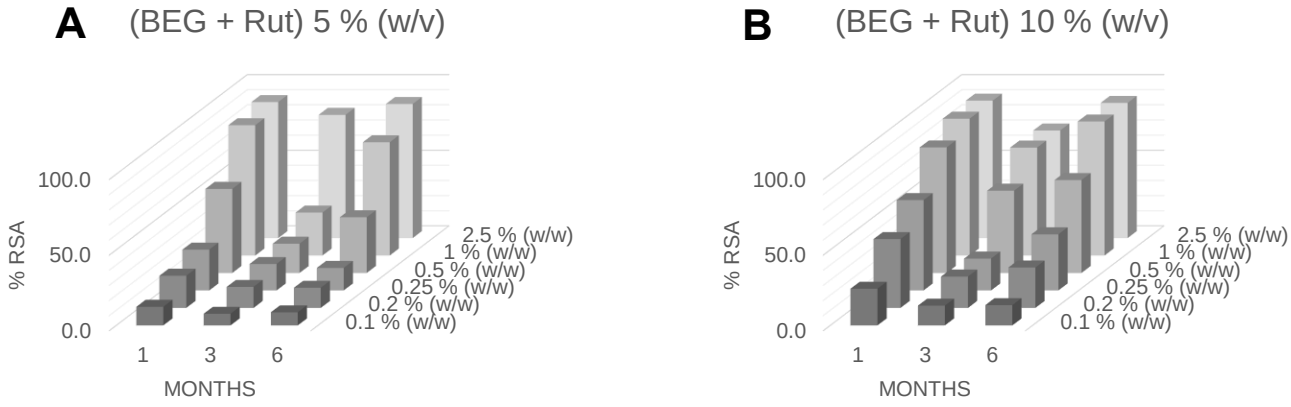

Figure A.5 - Variation of %RSA of the system Bet:EG (BEG) with rutin (Rut) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.1 to 2.5 % in graph A and 0.1 to 8.2 % in graph B.

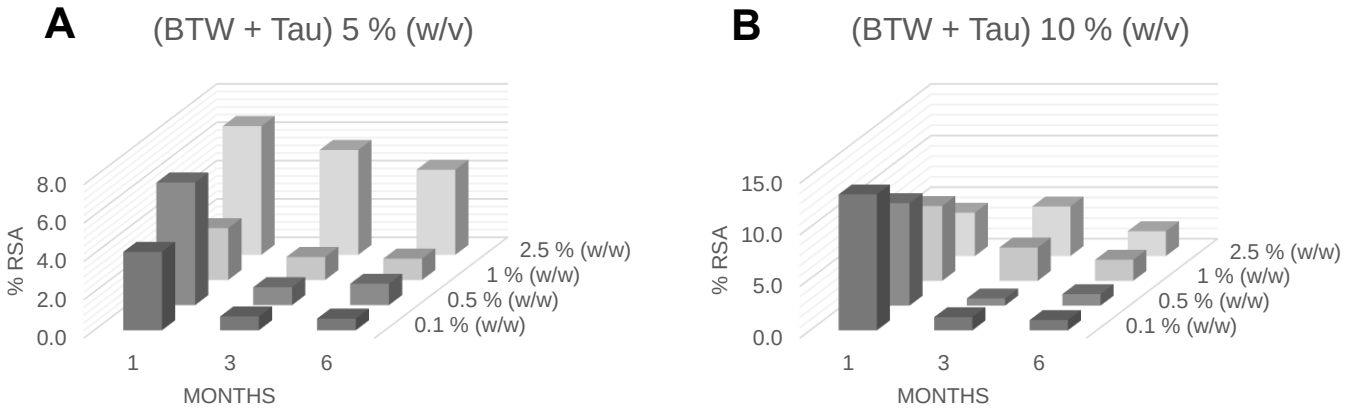

Figure A.6 - Variation of %RSA of the system Bet:Treh:W (BTW) with taurine (Tau) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.2 to 3.2 % in graph A and 0.0 to 5.5 % in graph B.

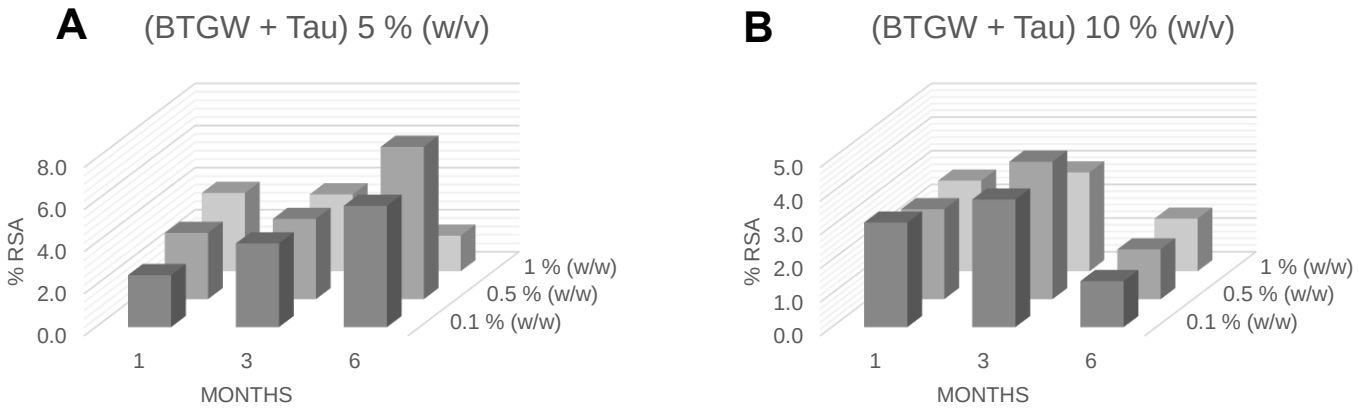

Figure A.7 - Variation of %RSA of the system Bet:Treh:Gly:W (BTGW) with taurine (Tau) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.1 to 1.3 % in graph A and 0.1 to 1.7 % in graph B.

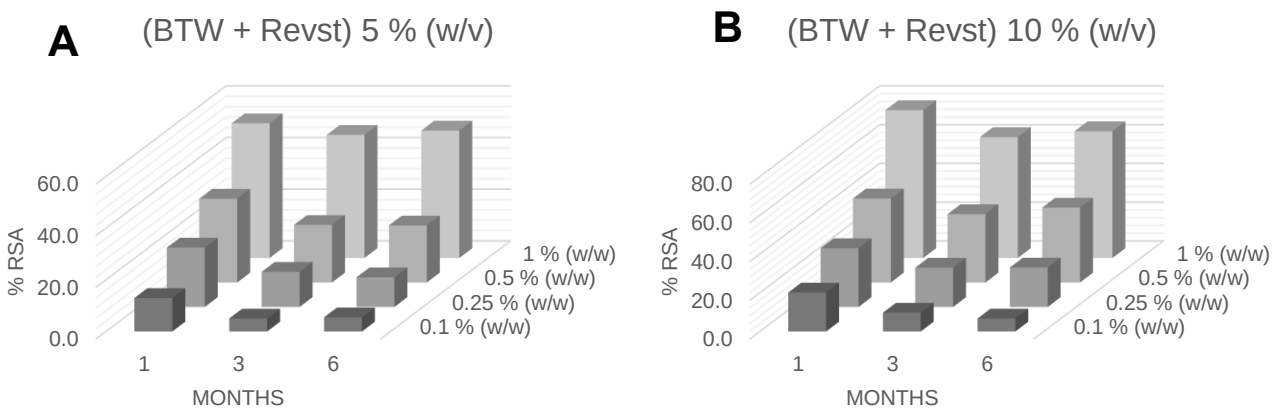

Figure A.8 - Variation of %RSA of the system Bet:Treh:W (BTW) with resveratrol (Revst) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.1 to 3.5 % in graph A and 0.2 to 3.3 % in graph B.

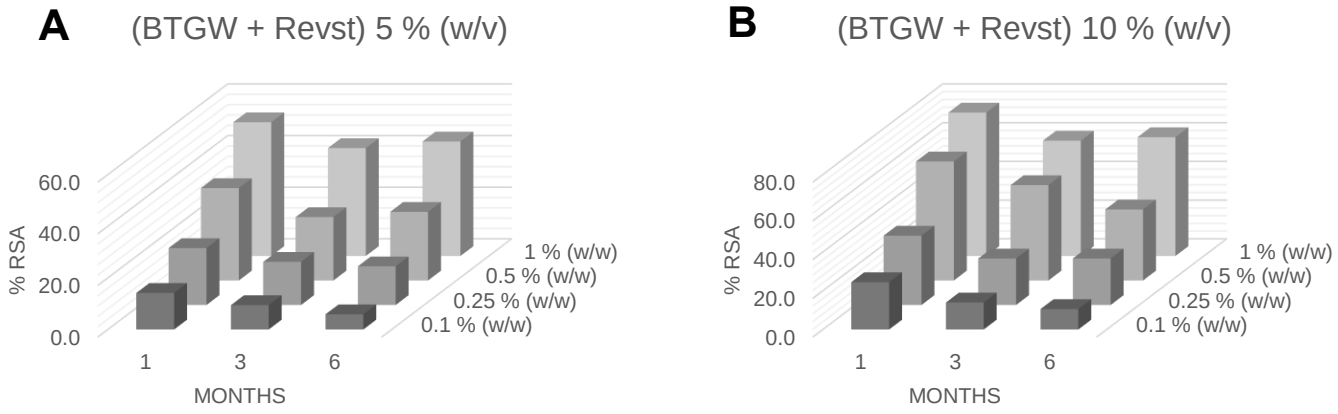

Figure A.9 - Variation of %RSA of the system Bet:Treh:Gly:W (BTGW) with resveratrol (Revst) at different concentrations (w/w) in solutions of 5 and 10% (w/v), A and B respectively, after 1, 3, and 6 months. Data indicated as mean and the SD varies from 0.4 to 2.4 % in graph A and 0.2 to 4.3 % in graph B.