Universidade de Lisboa
Faculdade de Farmácia

Biopharmaceutical Study of Quercetin Release from Matrix Pharmaceutical Forms

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Desde a década de 1930, os flavonoides têm vindo a suscitar interesse devido ao seu potencial para uso farmacêutico. A quercetina é o maior representante dos flavonóis, uma das subclasses de flavonoides, podendo ser encontrada em diferentes frutos, vegetais e bebidas. Este composto tem diferentes atividades bioquímicas e farmacológicas, uma vez que possui propriedades antioxidantes, anti-inflamatórias, antimicrobianas, anticancerígenas, anti-hipertensivas e hepato, cardio e neuroprotetoras. Para além disto, a quercetina promove a cicatrização de feridas e a proteção contra as radiações UV e tem efeitos anti-envelhecimento.

Apesar de todas estas atividades importantes, as propriedades físico-químicas da quercetina, como a baixa absorção no organismo, o metabolismo considerável e a alta ligação a proteínas, a fraca solubilidade em água, a baixa atividade intrínseca, a rápida eliminação e a baixa permeabilidade através da pele, resultam numa fraca biodisponibilidade. Para além disto, a quercetina é químio e termolábil e é rapidamente degradada quando exposta a meios alcalinos, luz e temperaturas elevadas, o que também limita a sua utilização.

Têm sido usadas várias abordagens para aumentar a solubilidade e a velocidade de dissolução da quercetina e, assim, melhorar a sua biodisponibilidade. As estratégias utilizadas incluem profármacos, nanoemulsões, ciclodextrinas, nanocristais, formulações lipídicas, nanopartículas de polímeros e sistemas de libertação transdérmica. Estas duas últimas estratégias, quando aplicadas à quercetina, têm mostrado resultados promissores.

Este estudo foi desenvolvido para investigar se a encapsulação de quercetina em nanopartículas de poli(2-hidroxipropil metacrilato) (PHPMA) e poli(2-hidroxietil metacrilato) (PHEMA), com diferentes razões PHPMA/PHEMA e com balanço hidrofílico-hidrofóbico controlado, iria afetar a os perfis de libertação do fármaco, a partir de adesivos transdérmicos. As nanopartículas foram incorporadas em pensos transdérmicos de polímeros hidrofílicos (poliacrilamida e polietileno glicol) e os perfis de libertação da quercetina foram seguidos utilizando espetroscopia UV. As condições dos testes de dissolução foram optimizadas.

Devido ao curto período de tempo disponível para realizar o trabalho, apenas foi possível traçar o perfil de dissolução da quercetina pura. No futuro, será necessário seguir os restantes perfis de dissolução, de forma a obter resultados mais conclusivos.

Palavras-chave: Quercetina; Biodisponibilidade; Testes de Dissolução; Adesivo Transdérmico

Abstract

Since the decade of 1930s, flavonoids have been gaining much interest due to their high potential for pharmaceutical usage. Quercetin is the primary representative of flavonols, a subclass of flavonoids. It can be widely found in different fruits, vegetables and beverages. This compound has many biochemical and pharmacological activities in relation to its antioxidant, anti-inflammatory, antimicrobial, anticancer, hepatoprotective, cardioprotective, antihypertensive and neuroprotective properties. When applied to the skin, quercetin also promotes wound healing, anti-ageing and UV protective effects.

Despite all these critical activities, quercetin's physicochemical properties, namely its low absorption, considerable metabolism and high protein binding, poor solubility in water, low intrinsic activity, fast clearance from the body and low permeation through the skin, result in low bioavailability. Furthermore, quercetin is chemo and thermolabile and degrades rapidly when exposed to alkaline media and light, which limits its usage.

Several approaches have been used to enhance quercetin's solubility and dissolution rate and, therefore, increase its bioavailability: prodrugs, nanoemulsions, cyclodextrins, nanocrystals, lipid formulations, polymeric nanoparticles and transdermal delivery systems. Among these, the last two ones have shown promising results when used for quercetin.

This study was developed to investigate the potential of quercetin encapsulation in poly(2-hydroxypropyl methacrylate) (PHPMA) and poly(2-hydroxyethyl methacrylate) (PHEMA) nanoparticles with different PHPMA/PHEMA ratios and controlled hydrophilic-hydrophobic balance, as a transdermal delivery system. Quercetin loaded nanoparticles were incorporated into hydrophilic matrixes based on hydrophilic polymers (polyacrylamide, polyethylene glycol) and the kinetics of quercetin release were studied. The drug release profiles were traced using dissolution tests and quantified by UV spectroscopy. The dissolution tests conditions were optimised.

Due to the short time that we had for carrying out the laboratory work, it was only possible to trace the dissolution profile of pure quercetin. In the future, it will be necessary to follow the remaining dissolution profiles in order to obtain more conclusive results.

Keywords: Quercetin; Bioavailability; Dissolution test; Transdermal Patch

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Abbreviations

UV	Ultraviolet
IUPAC	International Union of Pure and Applied Chemistry
TNF- α	Tumour necrosis factor-α
IL-1α	Interleukin 1-α
PKC	Phospho-protein kinase C
COX	Cyclooxygenase
LOX	Lipoxigenase
NF-kB	Nuclear factor-kappa B
AP-1	Activator protein 1
MAPK	Mitogen-activated protein kinase
NOS	Nitric oxide synthase
CRP	Reactive C-protein
HTLV-1	Human T-lymphotropic virus 1
JEV	Japanese encephalitis virus
AD	Alzheimer's disease
Aβ	Amyloid-β
BACE-1	β-active site cleavage enzyme-1
GSH	Glutathione
HIV	Human immunodeficiency virus
PBS	Phosphate-buffered saline
PHPMA	Poly (2-hydroxypropyl methacrylate)
PHEMA	Poly (2-hydroxyethyl methacrylate)
HEMA	2-hydroxyethyl methacrylate
HPMA	2-hydroxypropyl methacrylate

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1 Introduction

1.1 Flavonoids

Flavonoids are the biggest group of secondary metabolites synthesised mainly by higher plants (1,2). This important class of natural products can be widely found in fruits, vegetables, seeds, nuts and certain beverages, such as tea and wine. In the plant kingdom, they have several essential functions, such as pigmentation and protection against harmful UV radiation (2). Flavonoids can also act as chemical messengers, physiological regulators and cell cycle inhibitors (3) and they have antiviral, antibacterial and antioxidant properties, as well (2).

The general structure of these polyphenolic compounds includes a fifteen-carbon skeleton that comprises two benzene rings connected by a heterocyclic pyrene ring (Figure 1) (1). There are more than four thousand types of flavonoids in nature (4) that can be subdivided into several classes based on structural characteristics, such as the C-ring carbon to which the B-ring is attached, the degree of unsaturation and oxidation of the C-ring and the pattern of substitution of the benzene rings. The existing subgroups are flavones, flavonols, flavanones, isoflavonoids, flavanols or catechins, anthocyanins and chalcones. Each one of these subgroups has its own set of activities and benefits (3).

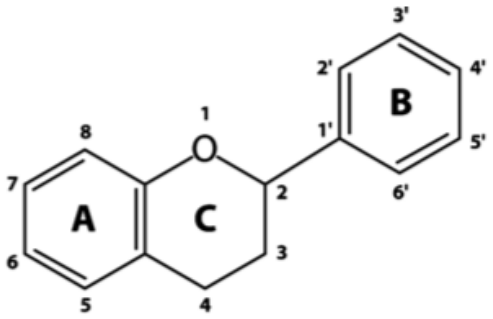


Figure 1 – Basic skeleton structure of flavonoids. Represented here are the two benzene rings (A and B) and the connecting heterocyclic pyrene ring (C). Adapted from: Zakaryan H, Arabyan E, Oo A, Zandi K. Flavonoids: promising natural compounds against viral infections. Arch Virol. 2017;162(9):2539-51.

The biological activity of flavonoids was first demonstrated in 1938, by Albert Szent-Györgyi. Nowadays, they are associated with a broad spectrum of health-promoting effects, thus being an indispensable part of a variety of nutraceutical, pharmaceutical, medicinal and

cosmetic applications. These effects are a result of their antioxidative, anti-inflammatory, antimutagenic, anticarcinogenic, antibacterial, antiviral, antiallergic, neuroprotective (1,3) and hepato-protective properties (4), in association with their low toxicity to the human body (5).

The primary clinical administration method of flavonoids is the oral administration; however, the majority of these compounds has limited oral bioavailability, resulting from their poor solubility in water, low permeability and poor stability. Furthermore, flavonoids are also sensitive to the various physical and physiological environments, probably leading to degradation and biotransformation during storage and systemic circulation, and consequently, limiting their efficacy when administered orally (6).

1.1.1 Classes of Flavonoids

As mentioned above, flavonoids can be subdivided into various classes (Figure 2) according to their different structural characteristics.

1. Flavones, such as luteolin, apigenin and tangeritin, can be widely found in leaves, flowers and fruits (3). They are different from other flavonoids because they have a double bond between C2 and C3, lack substitution at C3, and are oxidised at C4 (7).

2. Flavonols, the most common and largest subgroup of flavonoids, are characterised by the presence of a hydroxyl group at the C3 carbon position. They can be found in a variety of fruits and vegetables, as well as in tea and in red wine. The most known flavonols include kaempferol, quercetin, myricetin and fisetin. Their intake is linked to a wide range of health benefits since flavonols have vasorelaxant, antioxidant, antiproliferative, neuroprotective and anti-inflammatory properties (5,8).

3. Flavanones (e.g. hesperetin, naringenin and eriodyctiol) are usually present in fruits, such as oranges, lemons and grapes (3). They are associated with several different health benefits, as a consequence of their free radical scavenging properties, which lead to interesting pharmacological effects as antioxidant, anticancer, antimicrobial, anti-inflammatory, blood lipid and cholesterol-lowering agents (9). Flavanols, also known as catechins, are derivates of the flavanones and may be found in fruits, such as cocoa and red grapes. Their structure is characterised by having a hydroxyl group bound to position 3 of the C ring and by the absence of a double bond between positions 2 and 3, which is otherwise present in many flavonoids.

This subgroup is highly diversified since the compounds can be multisubstituted (3). These substances have been associated with improved cardiovascular health (10) and act as antioxidant, anti-inflammatory and anticarcinogenic agents (11).

4. Isoflavonoids (e.g. genistein and daidzein) have limited distribution in the plant kingdom since they are exclusively found in soya beans and other leguminous plants. Nevertheless, they have shown an enormous potential to induce hormonal and metabolic changes and, therefore, fight several different diseases (3), such as breast cancer, obesity and osteoporosis (12).

5. The subgroup of anthocyanins includes cyanidin, delphinidin and malvidin, among others. These are pigments responsible for the bright colours of plants, flowers and fruits, especially berries (3). Chemically, they are phenolic compounds with two benzene rings linked by a three-carbon chain. The study of anthocyanins is gaining interest because of their potential health benefits as antioxidants, helping to prevent neuronal and cardiovascular diseases, cancer, diabetes and inflammation, among others (13).

6. Chalcones, also referred to as open-chain flavonoids, are characterised by the absence of the C ring of the basic flavonoid skeleton structure (3), resulting in a three-carbon unsaturated system connecting the two aromatic rings. Phlorizin, arbutin and phloretin are some examples of members of this group. Different pharmacological activities, such as anti-inflammatory and anti-neoplasia, have been associated with chalcones (14).

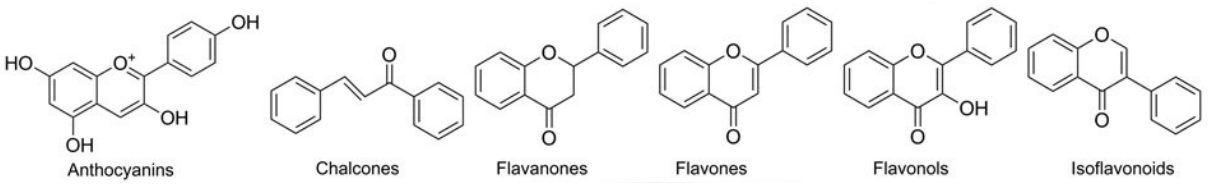


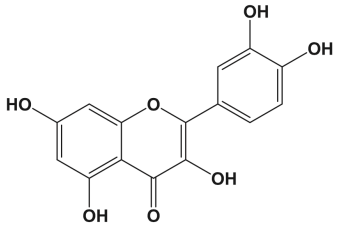
Figure 2 – The subclasses of flavonoids. Represented here are the basic skeletons of the major flavonoids' subclasses. Adapted from: Panche AN, Diwan AD; Chandra SR. Flavonoids: An overview. J Nutr Sci. 2016;5.

1.2 Quercetin

Quercetin, also known as 3,3',4',5,7-pentahydroxyflavone, is the principal representative of the flavonol subclass of flavonoids (15) and it was first isolated in 1936, by Szent-Gyorgyi (16). This compound is present in several fruits and vegetables, such as onions, broccoli, kale, tomatoes, apples and berries and in beverages, like tea and red wine (4,17).

As a member of the flavonols' subclass, quercetin's structure consists of a flavone (2-phenyl-1-benzopyran-1-one) backbone with a hydroxyl group attached to positions 3, 5, 7, 3' and 4' (18,19). Quercetin's chemical structure and physicochemical properties are presented in the following Table 1.

Table 1 - Quercetin physicochemical properties (15,20).

Chemical Structure	
Molecular Formula	$C_{15}H_{10}O_7$
Molecular Weight	302.2 g/mol
Chemical Name (IUPAC)	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one
Solubility	Low solubility in water Soluble in ethanol, acetone, pyridine and acetic acid Very soluble in ether and methanol
Physical Description	Yellow needles or powder
Polymorphism	Three polymorphic forms

In plants, quercetin is synthesised from the amino acid phenylalanine, which is converted to 4-coumaroyl-Coenzyme A, in a series of steps known as the general

phenylpropanoid pathway. One molecule of 4-coumaroyl-Cenzyme A is then added to three molecules of malonyl-Coenzyme A to originate tetrahydroxychalcone, by the action of a 7,2'-dihydroxy-4'-methoxyisoflavonol synthase. Afterwards, chalcone isomerase converts tetrahydroxychalcone to naringenin, flavonoid 3'-hydroxylase turns the latter into eriodyctiol, and this is subsequently converted to dihydroquercetin by flavanone 3-hydroxylase. Finally, dihydroquercetin is converted into quercetin, by flavonol synthase (16).

This flavonol has raised much attention, since it has shown a broad spectrum of biochemical and pharmacological activities (Figure 3), related to its antioxidant, anti-inflammatory, antimicrobial, antitumor and cardioprotective properties, among others (15,21). Quercetin can also be of help in the treatment of diseases such as glaucoma, promotes wound healing and protects the skin against UV radiation (22).

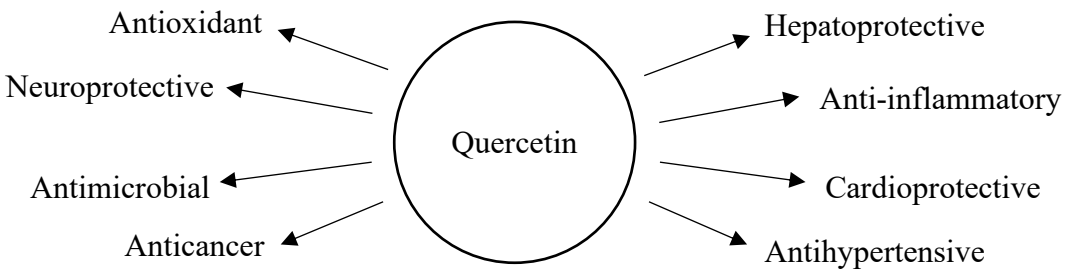


Figure 3 – Schematic showing of the multiple biological activities of quercetin. Adapted from: Cai X, Fang Z, Dou J, Yu A, Zhai G. Bioavailability of Quercetin: Problems and Promises. Curr Med Chem. 2013;(20):2572-82

1.2.1 Properties

1.2.1.1 Antioxidant Activity

The oxidative stress caused by free radicals and reactive oxygen species, either with an endogenous or exogenous origin, is continuously threatening body cells and tissues. This cellular damage can lead to different pathological conditions, such as diabetes, cancer, cardiovascular disease, neurodegenerative disorders and to ageing. The mechanisms whereby these free radicals affect cellular functions are not fully understood, but lipid peroxidation seems to have an important role, causing cell membrane damage, and then leading to osmotic pressure changes, cell swelling and death. Free radicals can also trigger an inflammatory process, contributing to tissue damage (3).

Quercetin is considered to be a potent antioxidant (20). Its antioxidant activity is connected to the presence of two antioxidant pharmacophores (e.g. the catechol group in ring B and the hydroxyl group in position 3 of the A ring), which are capable of scavenging free radicals and reactive oxygen species, having an additive effect to the endogenous scavenging pathway (3,20,23). Quercetin's scavenging mechanism is the result of the oxidation of its hydroxyl group by the free radicals, resulting in a more stable and less reactive radical (24). Other mechanisms related to quercetin's antioxidant activity include its effect on the incrementation of glutathione (GSH) levels, preventing free radicals' formation, the ability to inhibit the oxidative enzymes, acetyl and butyrylcholinesterase, and the effects on several signal transduction pathways (20,25).

1.2.1.2 Anti-inflammatory Activity

Generally speaking, inflammation is defined as the defence mechanism of our organism against an injury or infection, following physical trauma, contact with pathogenic organisms or with corrosive substances, as well as biological factors, such as oxidative stress. Many studies have shown that quercetin can act as a long-lasting anti-inflammatory agent (3,23).

Some *in vitro* studies have acknowledged that quercetin inhibits the development of tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α), which are essential compounds of the inflammatory process (19,23). By inhibiting calcium influx and phospho-protein kinase C (PKC), it has also been shown to inhibit the release of pro-inflammatory cytokines, tryptase and histamine from human umbilical cord blood-derived mast cells (19). Beyond this, quercetin has an inhibitory activity against the expression of the inflammation-producing enzymes cyclooxygenase (COX) and lipoxygenase (LOX), nuclear factor-kappa B (NF-kB), activator protein 1 (AP-1), mitogen-activated protein kinase (MAPK), reactive nitric oxide synthase (NOS) and reactive C-protein (CRP) (20,26).

1.2.1.3 Antimicrobial Activity

Quercetin has shown antiviral activity against several viruses, such as the human T-lymphotropic virus 1 (HTLV-1), the Japanese encephalitis virus (JEV) and human immunodeficiency virus (HIV). Moreover, it can suppress dengue virus type 2 and hepatitis C virus by inhibiting the non-structural protein 3 protease (20,23). Quercetin's antiviral activity results from its capability to affect the replication of some RNA and DNA viruses (27).

Quercetin has also demonstrated *in vivo* and *in vitro* antibacterial activity among different strains of bacteria, including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Staphylococcus aureus* and *Escherichia coli* (20,23).

Finally, the inhibitory effects of quercetin against various protozoan parasites, such as *Toxoplasma*, *Trypanosoma*, *Leishmania*, *Theileria* and *Babeisa* have been described in different reports (23). Besides, it was demonstrated that the activity against *Toxoplasma gondii* is due to the prevention of the heat shock protein 90, 70 and 27 synthesis and consequent suppression of bradyzoite development (28).

1.2.1.4 Anticancer Activity

Even with the technological and pharmaceutical evolution, malignancies continue to be a significant global concern. Cancer treatment methods include surgery, radiotherapy and chemotherapy, yet herbs have been used as complementary therapy agents for years (4). The anticancer activity of quercetin has been described both *in vitro* and *in vivo*. In *in vitro* experiments, the anticancer efficacy was determined by the prevention of angiogenesis in tamoxifen-resistant cancer, while the *in vivo* efficacy was associated with quercetin's antioxidant properties (23).

It was demonstrated that quercetin has the capability of preventing the proliferation of different types of malignant tumours, such as prostate, breast, lung, cervical, colon and liver cancers. It acts by various mechanisms, including cellular signalling, binding to cellular receptors and proteins, and inhibiting enzymes responsible for carcinogen activation (4).

Furthermore, Rauf et al. reported that quercetin had synergistic effects when used together with some chemotherapeutic agents, like cisplatin (4). It has also been documented that quercetin is able to increase the chemosensitivity of breast cancer cells to doxorubicin, thus preventing cell propagation and invasion. Beyond this, it was shown that quercetin up-regulates miR-146a and inhibits the NF-kB pathway, supressing the proliferation of human breast cancer cells and inducing human colon cancer cells apoptosis, respectively (29,30).

1.2.1.5 Hepatoprotective Activity

In vivo studies have described that quercetin increases the activity of heme oxygenase 1 by lowering the plasma concentrations of alanine aminotransferase and stimulating its

hepatoprotective activity. It was also shown that quercetin has the ability to treat ethanol-induced oxidative damage in rat hepatocytes, suggesting that this molecule can be considered a hepatoprotective natural product (23).

1.2.1.6 Cardioprotective and Antihypertensive Activity

The presence of reactive oxygen species has been associated with different disorders, such as diabetes, atherosclerosis, ischaemic heart disease, hypertension and heart failure (31). The antioxidant and anti-inflammatory characters of quercetin are gaining interest since they lead to the reduction of reactive oxygen species levels, lowering the risk of cardiovascular pathology (25,32).

On the other hand, an antihypertensive effect of quercetin was suggested after the observation of a dose-dependent reduction in blood pressure, when quercetin was given chronically to several spontaneously hypertensive rats (31). Besides, it was observed that the blood pressure of patients with hypertension was reduced after they had a regular intake of quercetin (33,34).

1.2.1.7 Neuroprotective Activity

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease, and multiple sclerosis, are characterised pathologically by a progressive loss of neurons. The leading causes of this degeneration include oxidative stress, deposit of protein aggregates, neuroinflammation, impaired mitochondrial function, induction of apoptosis and autophagy modification. Quercetin seems to be able to modulate some of these critical cellular processes (35) and its neuroprotective action has been raising attention, especially against Alzheimer's disease (AD).

AD, the most prevalent form of dementia, is a chronic neurodegenerative disease, characterised by memory loss, cognitive deficits and central nervous system inflammatory processes. A free radical imbalance is responsible for oxidative stress associated with the establishment of neurodegenerative disorders. In AD, quercetin's antioxidant properties contribute to the reduction of the prevalence of oxidative damage in the brain (23).

It is also known that AD is due to the accumulation of amyloid-β (Aβ) peptides, producing neurofibrillary tangles in the brain (3). Studies have shown that quercetin could

reduce the production of Aβ protein, via an NF-kB-dependent mechanism. By inhibiting the activation of NF-kB, quercetin promotes the down-regulation of the expression of BACE-1, an enzyme responsible for the production of Aβ peptides. With this mechanism, quercetin is able to lower Aβ peptides production and, therefore, alter the evolution of AD (36).

Beyond this, quercetin and other flavonoids act in the vascular system, altering cerebrovascular blood flow and, consequently, causing neurogenesis, angiogenesis and neuronal morphology modifications (37).

1.2.2 Toxic Side Effects and Drug Interaction

Quercetin is considered to be a safe compound without carcinogenic effects in several *in vivo* animal studies. The teratogenic activity has not been deeply analysed, but some *in vitro* studies suggest that quercetin can have a mildly negative impact on embryo development since it resulted in a small increase in the incidence of malignancies (20).

In human clinical trials, quercetin was well-tolerated and did not originate changes on serum electrolytes, kidney and liver function parameters, or blood cell counts. However, when high-dose IV quercetin was used in patients with compromised health, nephrotoxicity was reported (23).

Regarding drug interactions, it is known that quercetin is contraindicated in conjunction with fluoroquinolone antibiotics. Additionally, by acting as an inhibitor of CYP3A4, CYP1A2 and CYP2A6, xanthine oxidase and N-acetyltransferase, quercetin may increase the serum concentration of drugs that are metabolised by these enzymes, when administered simultaneously (20,23).

1.2.3 Bioavailability

By definition, bioavailability is the rate and extent to which a drug product becomes available and reaches the site of drug action. It depends mainly on the solubility, permeability, biotransformation and stability of the drug (38). Quercetin has a very low bioavailability, due to its poor absorption, considerable metabolism and high plasma protein binding, low aqueous solubility, low intrinsic activity and rapid clearance from the body (19,39,40). These

characteristics, alongside with its chemo and thermolability and its rapid degradation and discolouration when exposed to alkaline media and light, limit its pharmaceutical usage (23,41).

The low water-solubility of quercetin is a problem that has to be surpassed in order to make its usage more effective. Different approaches have been explored to enhance the solubility and dissolution rate of quercetin, in order to improving its bioavailability (6). Some of these strategies, which have been shown to be helpful include prodrugs and drug delivery systems, such as inclusion complexes, nanocrystals, emulsions, liposomes and phospholipid formulations, polymer nanoparticles and micelles (42).

1.3 Dosage Forms and Delivery Systems

Through the years, different dosage forms and delivery systems of quercetin have been used. Some of them are more effective than others. The more conventional dosage forms have shown some disadvantages, promoting the development of novel delivery systems. The latter allow quercetin to be delivered in more efficient and safe ways (38).

1.3.1 Conventional Delivery

The most conventional dosage forms include tablets, capsules and oral liquids. Oral administration of drugs is the most commonly route used when systemic effects are desirable. It is estimated that 90% of the active ingredients used in systemic therapy are administered by this route (43). These forms have the considerable advantage of having a formulation process that is relatively easier and less expensive, when compared to newer dosage forms and delivery systems. Nonetheless, the administration of quercetin through this type of dosage forms has shown some major inconveniences that originate loss of efficacy. This is mainly due to the first pass-effect and instability of quercetin in the gastrointestinal tract, resulting in a low bioavailability (38,44).

Conventional dosage forms for dermal application include gels, ointments, creams and emulsions. The aqueous gels are known for the fast release of the drug, while oily formulations provide prolonged release. Quercetin has been formulated in emulsions, because its low water solubility requires the presence of a lipid phase, despite aqueous formulations being more skin-

friendly and comfortable to apply. Emulsions containing a higher lipid content have proven to be more effective in delivering quercetin to the skin (22).

1.3.2 Prodrugs

Prodrugs are pharmacologically inactive substances that are converted in the body into pharmacologically active drugs. In drug research and development, these delivery systems are often designed to improve the drug's bioavailability and the therapeutic efficacy. The activation of the parent drug can occur via different chemical or enzymatic processes (42).

Since prodrugs can increase water solubility, enhance absorption and membrane permeability, as well as stabilise the effects, different strategies have been used to prepare quercetin prodrugs. Carboxyesters, sulfonates, esters of inorganic acids, acetals and carbamates have been shown to successfully enhance quercetin's water solubility and bioavailability (44). Likewise, quercetin-amino acid conjugates have been synthesised and their properties, including water solubility, stability and cell permeability were estimated. Remarkably, quercetin prodrugs with aspartic and glutamic acid revealed an increased water solubility, when compared to pure quercetin (45).

Although quercetin prodrugs have a high potential for improving bioavailability, intestinal metabolism, due to the degradation before absorption, is still limiting the drug's effectiveness at the site of action (6). The use of these prodrugs is also limited by the fact that few *in vivo* studies have been performed. Accordingly, further studies on this subject are necessary (44).

1.3.3 Nanoemulsions

Nanoemulsions are dispersions which comprise an oily phase, an aqueous phase and surfactants, and are isotropic and thermodynamically stable (46). These emulsions are characterised by some favourable properties: its nanometric size (diameter varying from 1 to 100 nm), transparency, low viscosity, high solubilisation capacity; they protect the drugs from degradation, hydrolysis and oxidation and are easy to prepare (42).

Several nanoemulsions were made with quercetin as the active ingredient, resulting in significantly increased water solubility and absorption (47–49). However, in some cases, the bioaccessibility and bioavailability of quercetin were relatively low, even after the production of the nanoemulsion (42).

1.3.4 Cyclodextrins

Cyclodextrins are cyclic α (1-4) linked glucose oligomers that have characteristic shape and dimensions. Because of their geometry, their hydrophobic internal cavity and their hydrophilic external faces, these molecules can easily form complexes that allow an increase in water solubility of poorly soluble drugs and, thus, enhance their bioavailability and stability (38,50).

Cyclodextrins have been widely used in multiple pharmaceutical dosage forms (oral, parenteral, dermal, ocular, nasal, rectal, sublingual) to modify the physical, chemical and biological properties of guest molecules through the formation of inclusion complexes (51).

Quercetin cyclodextrin solid inclusion complexes have been prepared and revealed an enhancement of the solubility and dissolution rate. This was shown by dissolution tests that were performed with a paddle apparatus, in pH 7.4 phosphate buffer with 0.8% sodium lauryl sulphate at 37 ± 0.5ºC. The rotation speed used was 50 rpm (52). It was also demonstrated that there was an improved activity, at a much lower concentration when compared with pure quercetin. This brings up the possibility of reducing quercetin's dose, without affecting the therapeutic efficacy, when using cyclodextrin carriers (42).

1.3.5 Nanocrystals

Another delivery system that has been used with quercetin is nanocrystals. Nanocrystals have an enhanced ability to transport across barriers, such as cell membranes. Besides, drug nanocrystals have a greater dissolution rate due to a larger surface area. They can be used for parenteral, oral, dermal, ocular and pulmonary administrations (42).

Quercetin nanocrystals are prepared via high-pressure homogenisation, bead milling and cavi-precipitation. The dissolution behaviour of these nanocrystals can be estimated using the paddle apparatus, in pH 6.8 phosphate-buffered saline (PBS) with 5% ethanol at 37ºC ± 0.5ºC and with a rotation rate of 100 rpm. The concentration of drug in the withdrawn samples was determined at 370 nm using an ultraviolet spectrometer. Different studies verified that nanocrystals dramatically enhanced quercetin's dissolution rate and, consequentially, its bioactivity. This has been attributed to the increased surface area by virtue of the decreased particle size of the nanocrystals (41,53).

1.3.6 Lipid Formulations

Solid lipid nanoparticles are a drug delivery system that consists of particles with a hydrophilic shell and a hydrophobic lipid core. These nanoparticles are solid at room temperature (54).

Hydrophobic drugs can be encapsulated into the lipidic core, resulting in increased stability, biocompatibility and bioavailability, reduced degradation and toxicity, controlled release, target specificity and multiple possibilities of administration routes (oral, intravenous, pulmonary and transdermal). Despite having these advantages, solid lipid nanoparticles have shown a low compound loading capacity and leakage during storage, which limit its use. (42,54). These particles have been successfully used for the delivery of quercetin, enhancing the oral bioavailability of the drug (55).

On the other hand, nanostructured lipid carriers are a new generation of lipid nanocarriers that are stable, biocompatible and biodegradable and have high loading capacity. Quercetin can be easily encapsulated into these particles, being stable in the lipid core. Different studies with quercetin-loaded nanostructured lipid carriers suggested that they were able to increase the drug's aqueous solubility, improve its sustained release, reduce its biotransformation and metabolism, enhance its bioavailability and bioactivity and lower its toxicity (54). When a dermal administration was used, this delivery system also led to the promotion of the skin permeation and the increase of drug retention in the skin; nevertheless, it caused the weakening of the barrier function of the stratum corneum (56).

1.3.7 Polymeric Nanoparticles

In recent years, there has been a big focus on developing polymeric nanoparticles for drug delivery (38). These particles are an excellent way to deliver drugs with low water solubility, since their nano size can increase the absorption and bioavailability of those drugs (57). Polymers have also been used to encapsulate drugs, due to their biodegradability, biocompatibility, low toxicity and controlled and targeted drug delivery capability (42,57).

Different quercetin-loaded polymeric nanoparticles have been designed using poly-d,*l*-lactide, poly(lactic-co-glycolic acid), polyvinyl alcohol and polyethylene glycol, resulting in a controlled release of the drug from the particles, enhanced dissolution and effectiveness, when compared to the pure drug (57–60). The dissolution tests for these nanoparticles were performed in a simulated intestinal fluid (pH 6.8), using the paddle apparatus. The temperature and rotation rate were, respectively, 37 ± 0.5ºC and 100 rpm (59,60).

The effect of quercetin-loaded nanoparticles highly depends on the carriers and on the physicochemical properties of the delivery system. These characteristics can enhance quercetin's stability, bioavailability and target specificity (40).

Poly (2-hydroxyethyl methacrylate) (PHEMA) and poly (2-hydroxypropyl methacrylate) (PHPMA) are two polymers that have been successfully used to encapsulate different drugs, such as quercetin (61). Notwithstanding, their use as drug carriers for quercetin has not been reported.

PHEMA is a transparent polymer that can be easily synthesised, linking hydrophilic monomers of 2-hydroxyethyl methacrylate (HEMA) (62). In terms of its chemical structure, HEMA has one carbonyl and one hydroxyl group on each side chain. These groups, alongside with the α-methyl groups, are responsible for the functions of the monomer and its hydrolytic stability (63). The biological usage of PHEMA is limited by the fact that it has a low biodegradability, as its degradation products have a high molecular weight. Nonetheless, some studies have been able to surpass this disadvantage by reducing the molecular weight of these products (64).

PHPMA is a polymer synthesised from the monomer 2-hydroxypropyl methacrylate (HPMA), which has already some biomedical applications, including as an artificial skin and or as drug delivery systems. This polymer has a hydrophilic character, due to its own hydroxyl groups (65). Yet, when compared to PHEMA, this polymer has lower hydrophilicity (66).

Both PHEMA and PHPMA-based nanoparticles have shown to be suitable for pharmaceutical technology applications due to their properties, such as a high water content, non-toxicity, biocompatibility and suitability for controlled drug delivery (61).

Despite the numerous advantages already referred, this bioavailability enhancing strategy also has some limitations, such as the cost of production and the toxic side effects of this delivery system (40).

1.3.8 Transdermal Delivery

The skin is the largest organ of the body, representing 15% of the total adult body weight. The skin is a multifunctional organ with different functions in the human body. It acts as a barrier between the exterior environment and the organism, protecting it from physical, mechanical and microbial injuries. It is also responsible for temperature regulation, nerve sensation, injury repair and metabolism (67).

Transdermal delivery is a drug's route of administration wherein the active ingredients are delivered through the intact skin, to reach a systemic distribution (68). It is an attractive alternative to oral delivery of drugs. This type of drug delivery has many advantages, including prolonged release administration and the avoidance of the first-pass effect. It is also painless, non-invasive, inexpensive and can be self-administered, improving patient compliance. Nonetheless, this delivery system also has some disadvantages, the most significant challenge being that only a limited number of drugs can be administrated via this route (69).

Transdermal patches are an example of a transdermal delivery system where the drug is administered in the form of a patch that delivers the drug into the circulation, for a systemic effect. In matrix transdermal patch designs, the drug is incorporated in a solid polymer matrix (70).

These transdermal drug-delivery systems are an adequate method to administrate free radical scavengers since the skin is an important target root for oxidative stress-related diseases and wounding.

Quercetin's transdermal delivery has shown to be able to enhance the drug's absorption. Poly(lactic-co-glycolic acid) and hyaluronic acid nanoparticles were prepared to encapsulate quercetin and were then emulsified to create a nanoemulsion with a cell permeation enhancer

(71). Transdermal patches of quercetin were also successfully prepared, enhancing its anti-inflammatory activity (68). In these studies, the drug dissolution behaviour was analysed using the flow-through cell USP apparatus 4. The flow rate was maintained at 8 mL/min and the temperature at 32ºC. One millilitre samples were collected at predetermined time intervals and replaced by 1 mL of fresh medium. The quercetin concentration was determined using HPLC. These studies concluded that quercetin-loaded nanoparticles can be successfully delivered through the skin and that this method led to an enhancement of the drug's solubility and bioavailability (68,71).

2 Aim of the Study

This thesis is integrated into the investigation developed in the Laboratory on Structure and Properties of Polymers and in the Laboratory on Pharmaceutical Technology and Biopharmacy of the Faculty of Chemistry and Pharmacy of Sofia University, Sofia, Bulgaria.

The main goal of this study was to investigate the potential of quercetin encapsulation in polymeric nanoparticles with controlled hydrophilic-hydrophobic balance and its application as a transdermal delivery system.

To achieve this purpose, quercetin was encapsulated into nanoparticles from PHPMA, PHEMA and their copolymers, with variable hydrophilic-hydrophobic balances, i.e. with different PHPMA/PHEMA ratios.

The quercetin-loaded polymeric nanoparticles were then incorporated into hydrophilic matrixes based on hydrophilic polymers (polyacrylamide, poly(ethylene glycol)) and their dissolution profiles were investigated. Pharmacopeial dissolution tests for solid forms (Ph. Eur. method 2.9.3) and transdermal patches (Ph. Eur. method 2.9.4) were performed, the paddle apparatus was used, and testing conditions were optimized in terms of dissolution media composition, pH, quercetin concentration, temperature and rotation rate. Quercetin's dissolution profiles were determined using UV spectroscopy.

The final goal of the study was to reveal the relation between release profiles and polymeric nanoparticles composition used for the quercetin encapsulation, in order to draw conclusions on which of the tested strategies is the best to enhance quercetin's water solubility and bioavailability.

3 Materials and Methods

3.1 Materials

3.1.1 Reagents

Quercetin (QCT, hydrate, ≥ 95%) was purchased from Cayman Chemical Company and stored at 22ºC. All the reagents used for the buffered salt solution, incluing, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, phosphoric acid and Tween 80 were purchased from Merck KGaA, Darmstadt, Germany and were also stored at 22ºC during the course of the study. All the reagents mentioned here were used as received from the supplier, without further purification.

The deionised water used to prepare the buffered salt solution was purified using a Milli-Q water purification system with minimum resistivity of 18.0 MΩ.cm.

3.1.2 Apparatus

A magnetic stirrer plate MSH-300N (BOECO, Germany) with temperature controller was used to help dissolve all the components of the buffered salt solution, and the HI2020-02 edge® multiparameter benchtop pH meter (Hanna Instruments, Italy) was used to adjust the solution's pH.

In the preparation of the calibration curve, an ultrasonic apparatus with controlled heating UST 2.8-100 (Siel, Bulgaria) was used to help dissolving the quercetin into the buffered salt solution. The S-26 spectrophotometer (BOECO, Germany) was used to determine the absorbance of the different standard solutions for the calibration curve and the samples of the dissolution test. All the dissolutions tests were performed using the paddle apparatus PJ-3 Tablet Four-Usage Tester (SaintyCo, China) with controlled temperature and rotation speed.

3.2 Preparation of the buffered salt solution pH 7.2, containing 1% polysorbate 80

A concentrate of the buffered salt solution pH 7.2 (European Pharmacopoeia) was prepared by dissolving 8.0 g of sodium chloride R, 0.2 g of potassium chloride R, 3.18 g of disodium hydrogen phosphate R and 0.2 g of potassium dihydrogen phosphate R, in water R. The different components were added while the solution was in a magnetic stirrer plate with heating (Magnetic Stirrer MSH-300N, BOECO, Germany), so that it was easier to dissolve them and obtain a clear solution. Then water R was added until the final volume of 1000.0 mL was reached.

After that, 10 mL of Tween 80 were added to 990 mL of the previous solution. The pH was regulated by adding a phosphoric acid solution until pH 7.2. For the adjustment of the pH, a pH meter (HI2020-02 edge® Multiparameter Benchtop pH Meter, Hanna Instruments, Italy) was used.

3.3 Preparation of a calibration curve

To build the calibration curve for quercetin, a stock solution of quercetin with the concentration of 153 mg/L was prepared, by dissolving quercetin in the buffered salt solution pH 7.2, containing 1% polysorbate 80, using an ultrasonic apparatus (UST 2.8-100, Siel, Bulgaria). From this solution, nine standard solutions (Figure 4) were prepared. In order to prepare each one of the standard solutions, the previously prepared standard solution was diluted, resulting in the concentrations shown in Table 2**Erro! A origem da referência não foi encontrada.**. The absorbance was then determined at a wavelength of 371 nm (the maximum absorption wavelength of quercetin), using a spectrophotometer (S-26 Spectrophotometer, BOECO, Germany). The reference used in this determination was the buffered salt solution pH 7.2, containing 1% polysorbate 80.

Figure 4 – Preparation of the nine standard solutions.

Table 2 - Standard solution preparation for the calibration curve.

Standard Solution	Concentration (mg/L)
P1	249.6
P2	124.8
P3	62.4
P4	31.2
P5	15.6
P6	7.81
P7	3.91
P8	1.95
P9	0.98

3.4 Dissolution test

In vitro dissolution tests are important to enable prediction of the *in vivo* performance of the drug. One of the main goals of these tests is to obtain information about the drug-release characteristics of a particular pharmaceutical form. Compliance with these tests assures that

most of the active ingredient will be dissolved in an aqueous medium within an acceptable amount of time, when the preparation suffers some agitation.

In vitro dissolution tests were carried out according to the European Pharmacopoeia. The dissolution behaviour of quercetin was estimated using a dissolution apparatus with the paddle method (SaintyCo PJ-3 Tablet Four-Usage Tester). Samples containing an equal amount of quercetin (20 mg) were dispersed into 900 mL of buffered salt solution pH 7.2, containing 1% Tween 80. The temperature of the vessels was set at 32 ± 0.5ºC, to mimic the skin's temperature. The instrument was set to stir at a rotation rate of 50 rpm. At 5, 10, 30, 60, 90, 120, 150, 180 and 240 minutes, 5 mL samples of dissolution medium were withdrawn from each vessel, filtrated through 0.45 μm membrane filters (MF-Millipore Membrane Filter, Merck, Germany) and placed into vials. To compensate, an equal amount of blank medium was added immediately to maintain a constant volume following each sampling. The samples were then analysed at λ = 371 nm using a UV/Vis spectrophotometer (S-26 Spectrophotometer, BOECO, Germany). Every sample, except those withdrawn at the 5 minutes time stamp, were diluted to 25 mL so that the value of absorption would be within the range accepted for this method. The concentration of each sample was calculated using the calibration curve that was previously traced. Every single dissolution experiment was performed in triplicate. The dissolution test that was performed is presented in Figure 5.

Figure 5 – Dissolution testing of pure quercetin.

3.5 UV/Vis Spectrophotometry

Absorption spectrophotometry is a method used for the qualitative and quantitative determination of molecules present in a solution. Thus, the method is used to determine concentrations of a molecule in solution, using the Beer-Lambert law. This law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length.

In this study, this method was used to determine the absorbances of the different solutions of the calibration curve. The quartz cells (length of 1 cm) were filled with each solution at a time and placed inside the spectrophotometer (S-26 Spectrophotometer, BOECO, Germany). The values of the absorbances were measured and registered.

The UV/Vis spectrophotometry was also used to determine the absorbances of the various samples withdrawn from the vessels of the dissolution apparatus. Once again, the quartz cells were filled with each sample at a time, and the absorbances measured and registered. The concentration of quercetin in the samples was then determined, using the obtained values and the calibration curve equation.

4 Results and Discussion

4.1 Calibration curve

The preparation of the calibration curve allowed the validation of UV-Vis spectrophotometry method. We were able to determinate the range of concentrations where the method is linear, precise and exact. The first four standard solutions (P1-P4) did not fit this range, which meant that only the last five standard solutions were used for the construction of the calibration curve.

Table 3 – Absorbances of the standard solutions.

Standard Solution		Absorbance (λ = 371 nm)	Average ± SD
P5	5	0.960	0.940 ± 0.020
	5’	0.940	
	5’’	0.921	
P6	6	0.497	0.474 ± 0.022
	6’	0.471	
	6’’	0.453	
P7	7	0.262	0.243 ± 0.017
	7’	0.238	
	7’’	0.228	
P8	8	0.121	0.116 ± 0.004
	8’	0.113	
	8’’	0.115	
P9	9	0.101	0.084 ± 0.019
	9’	0.088	
	9’’	0.064	

The absorbance values of the standard solutions were measured in triplicate and are presented in Table 3.

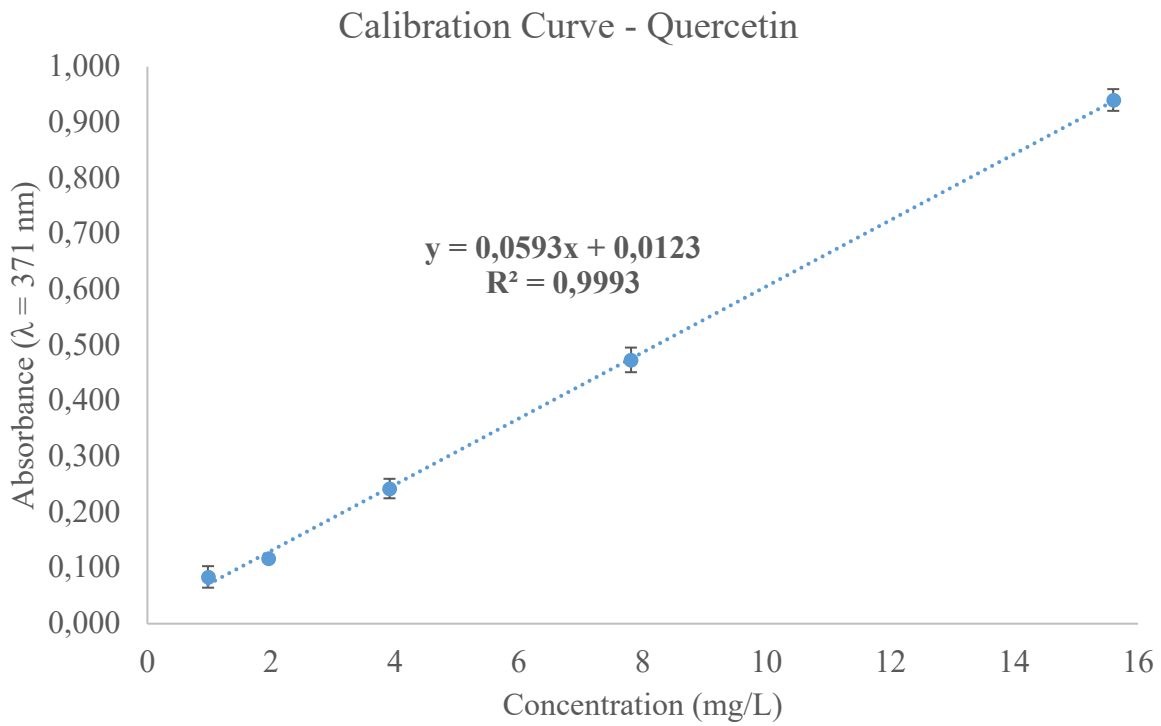


Figure 6 – Calibration curve of quercetin.

As it is shown in Figure 6, the calibration curve shows good values of correlation coefficient ($R^2 > 0.99$) between absorbance ($\lambda = 371$ nm) and concentration (mg/L), suggesting an acceptable linear relationship between the two variables and an excellent representation of the data, by the regression equation of the curve obtained. From the results, it was possible to conclude that the method is linear between the concentrations of 15.6 and 0.98 mg/L.

Due to these characteristics of the calibration curve, and for the interval of values in which the method is linear, the regression equation has been and will be used to determine the concentration of the samples withdrawn from the dissolution tests that have been and will be performed in the remainder of the study.

4.2 Dissolution study

The dissolution study was performed using the paddle apparatus. The absorptions of the samples withdrawn from the three vessels of the apparatus, as well as the concentrations of the samples, which were calculated using the calibration curve equation previously presented, are displayed in Table 4.

Table 4 - Absorbances and concentrations of the samples from the dissolution test.

Time (min)	Vessel	Absorbance (λ = 371 nm)	Average ± SD	Concentration (mg/L)	Average ± SD
5	1	0.924	0.798 ± 0.11	15.37	13.25 ± 1.85
	2	0.721		11.95	
	3	0.749		12.42	
10	1	0.192	0.179 ± 0.01	18.94	17.54 ± 1.41
	2	0.179		17.56	
	3	0.165		16.13	
30	1	0.212	0.211 ± 0.01	21.06	20.93 ± 1.02
	2	0.220		21.88	
	3	0.201		19.86	
60	1	0.223	0.220 ± 0.01	22.19	21.92 ± 1.00
	2	0.228		22.75	
	3	0.210		20.81	
90	1	0.228	0.239 ± 0.03	22.75	24.06 ± 3.24
	2	0.271		27.75	
	3	0.218		21.69	
120	1	0.246	0.226 ± 0.02	24.63	22.52 ± 1.87
	2	0.212		21.06	
	3	0.220		21.88	

150	1	0.220	0.213 ± 0.01	21.88	21.19 ± 0.64
	2	0.208		20.63	
	3	0.212		21.06	
180	1	0.223	0.214 ± 0.01	22.19	21.29 ± 0.81
	2	0.212		21.06	
	3	0.208		20.63	
240	1	0.240	0.221 ± 0.02	24.00	22.00 ± 1.73
	2	0.212		21.06	
	3	0.208		20.94	

The absorption values obtained for four of the samples – vessel 1 (5, 120 and 240 min) and vessel 2 (90 min) – were considered abnormally high. These results are probably due to the use of cuvettes that were not perfectly clean, when the absorption measurement was performed using the UV/Vis spectrophotometry method. This led to the calculation of concentrations that were not entirely accurate. Hence, the already referred values were not taken into account when tracing the dissolution profile of pure quercetin (Figure 7).

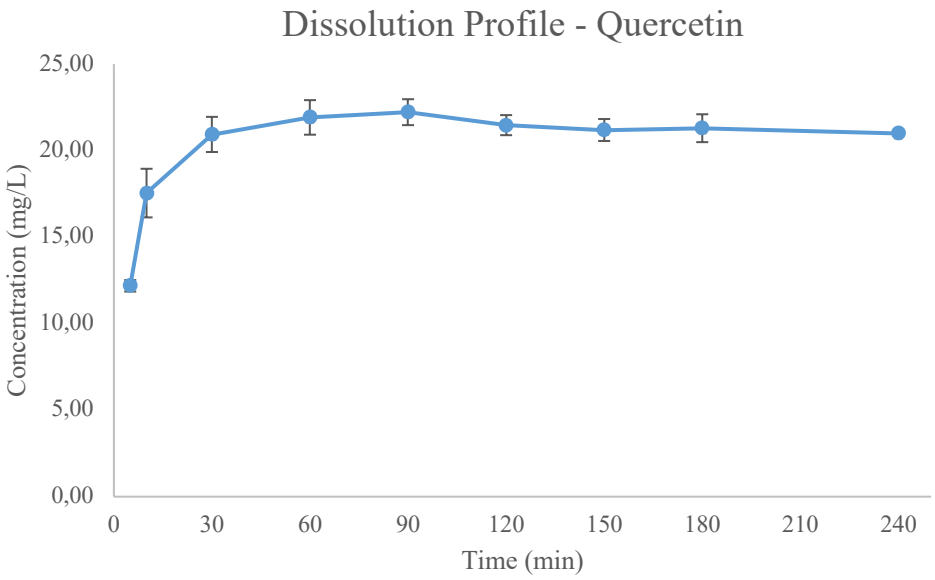


Figure 7 – Dissolution profile of pure quercetin.

This dissolution profile shows that, by the 60th minute of the test, the pure quercetin was 100% dissolved on the buffered salt solution pH 7.2 medium. From that point on, it is possible to observe that the concentration stayed practically constant. The variations observed are not significant and can be attributed to random measurement errors.

Since the results obtained from this test were considered valid, it is possible to infer that the testing conditions were optimised in terms of dissolution medium composition, pH, quercetin concentration, temperature and rotation rate. It is also possible to assume that this dissolution profile is a suitable means of comparison for the dissolution profiles that will be carried out in the future.

Unfortunately, the course of this study was interrupted by the COVID-19 pandemic crisis that led to the closing of the laboratories. Therefore, the initial results presented above were the only ones that were possible to obtain.

5 Futures Perspectives

The main goal of this work was to prepare transdermal patches with quercetin loaded PHPMA and PHEMA based nanoparticles and to compare their release profiles, in order to unveil which of these strategies was the best to enhance quercetin's bioavailability. Since the laboratories in which the studies were performed were closed after one month of the beginning of the work, due to the COVID-19 pandemic, the goal was not achieved. It was only possible to perform the preparatory work, the calibration curve for quercetin and the dissolution test for pure quercetin, which would be used as a reference for the comparison of the different dissolution and release profiles.

Hence, in the future, the intention is to perform the dissolution tests for the PHPMA and PHEMA nanoparticles with encapsulated quercetin, as well as for nanoparticles of their copolymers with different PHPMA/PHEMA ratios. This is going to be achieved using the paddle apparatus method that was described above, under the same conditions as for the pure quercetin testing. It is expected that the results of these tests will show that the dissolution of the quercetin nanoparticles will be easier than with pure quercetin. It is possible to predict this, since PHPMA and PHEMA are polymers that have already been successfully used to encapsulate hydrophobic drugs, enhancing their solubility in water and, therefore, increasing the velocity of dissolution, as shown in different previously referred studies.

We are also planning to evaluate the release profiles of quercetin from the polyacrylamide and poly (ethylene glycol) transdermal patches, using the European Pharmacopoeia method 2.9.4 to determine the dissolution rate of active ingredients of transdermal patches. According to this method, the dissolution tests for transdermal patches are performed using the paddle apparatus with the addition of a stainless-steel disk assembly, an extraction cell or a stainless-steel stirring cylinder. The method will be performed under the optimised conditions that were used for the pure quercetin dissolution test, namely the dissolution medium and its volume, rotation rate, temperature and sample withdrawal times.

It is known that the dissolution profile of a drug highly influences its topical bioavailability since with faster dissolution and higher solubility, a higher concentration gradient is generated between the formulation and the skin; thus, more of the dissolved drug will be absorbed, also allowing faster skin penetration.

For quercetin, its low rate of dissolution is a limiting factor for its skin absorption. Since it is expected that the transdermal delivery of PHPMA and PHEMA nanoparticles through transdermal patches are adequate strategies to improve quercetin's dissolution profile and considering that the initial tests have shown that these particles have been successfully loaded with quercetin, we expect to reach the conclusion that PHPMA and PHEMA nanoparticles are good perspective carriers in the formulation of transdermal systems for the delivery of quercetin.

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