UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

Ana Kovačič

DIPLOMSKO DELO

KOZMETOLOGIJA

Ljubljana 2017

UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

Ana Kovačič

IZDELAVA IN VREDNOTENJE NANOKRISTALOV KVERCETINA ZA DERMALNO UPORABO

PREPARATION AND CHARACTERIZATION OF QUERCETIN NANOCRYSTALS FOR DERMAL APPLICATION

Ljubljana 2017

Practical and theoretical part of my final thesis were made as a part of Erasmus exchange program in spring 2016. Materials, machines and supervision were provided by Faculty of biology and pharmacy, University of Cagliari, Sardinia.

GRATITUDE

I would like to express gratitude to my parents, friends and boyfriend for all the support and patience during my study and to all the professors on Faculty of pharmacy Ljubljana for sharing their knowledge. I would also like to thank my Italian mentor, Assoc. Prof. Dr. Sinico, for guiding me throughout the entire process of final thesis and to all the assistants on University of Cagliari to support and supervise me during my lab work. Special appreciation also goes to Assoc. prof. Dr. Petra Kocbek for her patience, kindness and help while guiding me through final stages of bachelor´s degree.

STATEMENT

I hereby declare that this final thesis was written by me under the supervision of Associate Prof. Dr. Petra Kocbek and co-supervision of Associate Prof. Dr. Chiara Sinico.

Ana Kovačič

COMMITTEE:

Head of committee: Prof. Dr. Aleš Obreza

Mentor: Assoc. Prof. Dr. Petra Kocbek

Co- mentor: Assoc. Prof. Dr. Chiara Sinico

Member of committee: Assist. Prof. dr. Urša Pečar Fonović

TABLE OF CONTENTS

ABSTRACT IV

RAZŠIRJENI POVZETEK VI

LIST OF USED ABBREVIATIONS X

1 INTRODUCTION 1

1.1 Nanocrystals 1

1.1.2 Production of nanocrystals 1

1.2 Hydrogel 5

Hydroxyethylcellulose (HEC) 6

1.3 Antioxidants 6

1.3.1 Quercetin 6

1.4 Skin 7

1.5 Photon correlation spectroscopy and laser Doppler anemometry 8

2 OBJECTIVES 9

3 MATERIALS AND METHODS 11

3.1 Materials 11

3.2 Equipment 11

3.3 Preparation of nanosuspensions 11

3.4 Characterization of nanosuspensions 14

3.5 Preparation of hydrogels 14

3.6 Preparation of hydrogel with quercetin nanosuspension 14

3.7 Preparation of quercetin hydrogel with coarse quercetin particles 15

3.8 Permeation experiments using Franz cells 15

3.9 Analysis of quercetin in pig skin layers 17

3.10 HPLC measurements 18

4 RESULTS AND DISCUSSION 19

4.1 Nanosuspension measurements 19

4.2 Penetration of quercetin in and through the skin... 24

6 CONCLUSIONS... 28

7 LITERATURE... 30

KAZALO SLIK:

Figure 1- Wet ball media milling device …………………………………………………………..12

Figure 2- Franz cell with marked receptor and donor compartment ………………………………16

Figure 3- Pig skin used for Franz cell experiment …………………………………………………16

ABSTRACT

The field of pharmacy is constantly improving and is seeking for new solutions, improvements and alternatives to already known methods and techniques. The situation is pretty similar in the area of cosmetic science, where scientists are trying to find the ways to satisfy the consumer´s needs and make cosmetic products as efficient as possible. One of the major concerns and challenges is, how to deliver active cosmetic ingredient at the right place (to specific target in the certain skin layer) and how to make it bioavailable and bioactive, so the effects would be visible or measurable. The cosmetic scientists are constantly developing new delivery systems and are searching for more suitable and effective ways for application. Furthermore, the researchers are oriented towards finding the solutions for application of non-soluble or poorly water soluble active cosmetic ingredients, since it is very difficult to incorporate them into the product, which would enable their bioactivity. One of the recent approaches is preparation of nanocrystals as delivery system for such active cosmetic ingredients. Nanocrystals are defined as nanosized particles of pure active ingredient without any matrix material with an average particle size less than 1000 nm (usually in the range between 200 nm and 500 nm). By decreasing the particle size, the total surface area is increased and therefore saturation solubility and dissolution velocity are increased and the compound bioactivity is usually improved. Nanocrystals, stabilized by surfactants or polymers, are usually dispersed in a liquid medium, thus forming a nanosuspension. In our case polysorbate 80 (Tween 80) and poloxamer 188 were used as stabilizers. There are plenty of techniques available for nanocrystal preparation. They are basically divided in three groups: bottom up methods, where nano-sized crystals of active ingredient are obtained from primary building blocks i.e. molecules, usually by precipitation process, top down methods, where micro sized or bigger particles become nano-sized by milling or high pressure homogenization process and combination of different methods. The method used in our study was wet milling process (top-down technique) which is commonly used due to its simplicity and possibility to be carried out in most laboratories. An aqueous suspension of coarse active ingredient, stabilized with surfactant or/and polymer is mixed with media milling beads at really high speeds. The active ingredient in form of nanosized particles is capable of reaching target site in the skin. Quercetin, natural flavonoid, investigated in our study, can thus provide its antioxidant activity at the target site. We wanted to prove these theoretical facts and show how coarse and nanosized quercetin penetrate differently in the skin. Therefore, the penetration depth and velocity were investigated using Franz cell experiment. By wet-milling

process we reduced the size of antioxidant particles (average size of particles in HEC hydrogel was 567.9nm +/- 6.1 and 369.9+/- 9.1 nm in Poloxamer 407 hydrogel), prepared the nanosuspensions with the most efficient investigated stabilizer (Tween 80 showed the best results) and incorporated it into a cosmetic dosage form i.e. the Poloxamer 407 and HEC hydrogel. The results showed that antioxidant in form of nanocrystals reaches deeper skin layers i.e. epidermis (2.0% in HEC hydrogel and 1.7% in case of Poloxamer 407 hydrogel) and dermis (1.3% in case of HEC hydrogel and 3.0% in case of Poloxamer 407 hydrogel), but does not penetrate through the full skin thickness and reaches the donor compartment *in vitro*. Quercetin in form of coarse particles stays mainly in *stratum corneum* or on the skin surface and reaches epidermis and dermis in negligible amounts (epidermis 0.8% in case of HEC hydrogel and 0.1% in case of Poloxamer 407 hydrogel; dermis 0.6% in case of HEC hydrogel and 0% in case of Poloxamer 407 hydrogel). Our hypothesis has been thus confirmed and our laboratory work showed that innovative technologies is a very promising, industrially applicable, approach for achieving better effectiveness of poorly soluble active cosmetic ingredients, such as quercetin.

RAZŠIRJENI POVZETEK

Farmacija se neprestano izboljšuje, napreduje ter konstantno išče nove izzive, nove rešitve, izboljšave in alternative že poznanim tehnikam, materialom ter končnim produktom. Podobna zgodba se odvija na področju kozmetologije, kjer strokovnjaki nenehno iščejo najbolj učinkovite rešitve za zadovoljitev potrošnikovih potreb. Ne le za zadovoljstvo pri uporabi, znanstveniki se ukvarjajo predvsem z izdelavo novih, izboljšanih dostavnih sistemov, ki bi kozmetično aktivne sestavine dostavili do tarčnega mesta oziroma do prave plasti kože, obenem pa ohranili biološko aktivnost sestavine in s tem dosegli želene rezultate. Ogromno raziskav se odvija na področju dostave v vodi težko topnih kozmetično aktivnih sestavin, kako jih vključiti v kozmetične oblike in hkrati zagotoviti njihovo stabilnost, učinkovitost in ustrezno prehajanje v kožo. Eden izmed novejših in bolj uspešnih pristopov je izdelava nanokristalov. To so nanometrski delci kozmetično aktivne sestavine brez ogrodnega materiala velikosti do 1000nm (najpogosteje v območju med 200 in 500nm). Z zmanjšanjem velikosti delcev se poveča njihova površina na enoto mase, s tem pa tudi reaktivnost, biološka aktivnost, topnost in hitrost raztapljanja ter oprijemljivost oziroma adhezija delcev na mesto nanosa izdelka, kar je pomembno pri končni dermalni aplikaciji. Disperzijo nanokristalov v tekočem mediju imenujemo nanosuspenzija. Metode izdelave nanokristalov v grobem delimo v dve skupini: metode od spodaj navzgor (″bottom-up″), kjer iz osnovnih gradnikov izdelamo delce kozmetično aktivne sestavine nanometrskih velikosti (npr. obarjanje) in metode od zgoraj navzdol (″top down″), kjer iz večjih delcev s pomočjo mletja ali homogeniziranja pod visokim tlakom izdelamo nanometrske delce. Včasih lahko zaradi različnih razlogov uporabimo kombinacijo različnih metod. V okviru diplomske naloge smo uporabili metodo mokrega mletja, ki za razliko od suhega mletja, omogoča izdelavo delcev nanometrske velikosti, hkrati pa je metoda izvedljiva v praktično vsakem laboratoriju, ki je opremljen z mlinom za mokro mletje. Poleg vodne disperzije kozmetično aktivne sestavine potrebujemo dodatek medija za mletje (majhne kroglice, ki omogočajo mehansko zmanjševanje velikosti delcev) in stabilizatorja, ki preprečuje združevanje delcev v agregate. Kot kozmetično aktivno sestavino smo uporabili kvercetin, ki je učinkovit antioksidant iz skupine flavonoidov. Nahaja se v najrazličnejšem sadju in zelenjavi (npr. čebula, jabolka, jagodičevje). V kozmetične namene se uporablja predvsem zaradi protivnetnih in antioksidativnih lastnosti, zaradi česar je učinkovit pri zaščiti in preprečevanju razgradnje elastičnih in kolagenskih vlaken, obenem pa plasti kože ščiti pred učinki UVB sevanja. Antioksidanti preprečujejo oksidativni stres, ščitijo

celice pred nevarnimi radikali in obnavljajo poškodovane celice. Kvercetin kot kozmetično aktivna sestavina ima lahko te učinke le na dermalni ravni, saj iz kozmetičnega izdelka ne sme prehajati v sistemski krvni obtok, kar je predpisano v Uredbi o kozmetičnih izdelkih.

Na začetku laboratorijskega dela smo naredili štiri različne formulacije nanosuspenzij kvercetina z različnimi koncentracijami kozmetično aktivne sestavine ter različnimi stabilizatorji: 3 % (w/w) nanosuspenzija kvercetina, stabilizirana s Poloksamerom 188 ali Polisorbatom 80; 5 % (w/w) nanosuspenzija kvercetina, stabilizirana s Poloksamerom 188 ali Polisorbatom 80. Na podlagi podatkov o povprečni velikosti delcev, polidisperznem indeksu in potencialu zeta smo za nadaljnje laboratorijsko delo uporabili najbolj optimalno formulacijo nanosuspenzije. Kot taka se je izkazala 3 % (w/w) nanosuspenzija kvercetina stabilizirana s Polisorbatom 80 s povprečno velikostjo delcev 316,3 nm, povprečnim polidisperznim indeksom 0,159 in potencialom zeta -38,2 mV. Za nadaljnje raziskave smo pripravili 12 paralelk opisane nanosuspenzije in jih združili v čaši, tako da smo dobili dovolj velik vzorec homogene nanosuspenzije za nadaljnje raziskave. Povprečna velikost delcev v tem vzorcu je bila nekoliko večja (517,1 nm), polidisperzni indeks (0,120) in potencial zeta (-40 mV) pa sta bila manjša. S tem vzorcem nanosuspenzije smo nato nadaljevali praktično delo.

Ker smo želeli inovativen dostavni sistem v obliki nanokristalov preizkusiti v realnem kozmetičnem izdelku, smo v ta namen izdelali dva različna hidrogela - enega s hidroksimetilcelulozo (HEC) in enega s Poloksamerom 407. Pri prvem smo uporabili HEC (2,5 %, w/w), jo raztrli v glicerolu (10 %, w/w), dodali zahtevano količino vode (87,5 %, m/m) in pustili približno pol ure, da se je ustvarila 3D rešetka tj. gel je nabreknil in dobil svoje značilnosti. Pri hidrogelu poloksamera 407 je bila izdelava še nekoliko lažja, saj smo poloksameru 407 (20 %, w/w) le dodali vodo (80 %, w/w) in pustili, da je gel nabreknil. Končno formulacijo hidrogela smo pripravili tako, da je le-ta vsebovala 15 % (w/w) nanosuspenzije kvercetina. Za primerjavo in kontrolo smo pripravili referenčno formulacijo hidrogela s kvercetinom, v katero smo vgradili izhodno uprašeno kozmetično aktivno sestavino.

Končni kozmetični izdelek smo uporabili za test s Franzovimi celicami. Z njim lahko ocenimo permeabilnost snovi preko umetne membrane ali biološkega tkiva (kože), koncentracijo snovi v posamezni plasti tkiva in hitrost prehajanja v in skozi tkivo. Statična Franzova celica je sestavljena iz donorskega dela, kamor nanesemo preizkušano snov, in receptorskega dela, kjer zbiramo snov, ki je prešla tkivo ali umetno membrano, obenem pa je

v receptorskem mediju fiziološka raztopina, ki simulira naravno okolje, in magnetno mešalo, ki omogoča nenehno mešanje medija. Med donorski in receptorski del smo vstavimo kožo, katere *stratum corneum* je obrnjen proti donorskemu delu, dermis pa je obrnjen proti receptorskemu delu. Najpogosteje uporabljena koža za tovrstne eksperimente je prašičja, saj ima podobno lipidno sestavo ter razporeditev in sestavo kolagenskih vlaken in žil, približno enaka pa je tudi njena debelina in debelina posameznih kožnih plasti.

Za test s Franzovimi celicami smo najprej ustrezno hidratirali kožo. Po 24ih urah smo na vsakega izmed šestih izrezanih delov kože nanesli 200 µL hidrogela HEC z nanosuspenzijo kvercetina. Vsakih 120 min smo fiziološko raztopino v receptorskem delu zamenjali s svežo in odvzet vzorec zamrznili na -20 °C. Enako smo naredili tudi za nanosuspenzijo v hidrogelu iz Poloksamera 407. Nato pa smo postopek ponovili še za kontrolna hidrogela s kvercetinom (v mikronizirani obliki). Shranjene vzorce receptorskih medijev smo nato liofilizirali in s tem pripravili suho stabilno trdno snov za analizo vsebnosti kvercetina .

Kožo smo po opravljenem testu odstranili ter jo ločili na *stratum corneum* (z adhezivnimi lističi); preostali epidermis in dermis pa smo ločili s pomočjo skalpela. Vsako plast smo nato razrezali na majhne koščke ter jih spravili v metanol, da bi z uporabo sonifciranja ekstrahirali antioksidant (kvercetin v vodi namreč ni topen). Viale smo napolnili z vzorci ter vsebnost kvercetina v posameznih plasteh kože določili s metodo HPLC.

Predpostavljali smo, da bo kvercetin v obliki nanosuspenzije zaradi manjše velikosti delcev difundiral hitreje, globlje in v večji količini v kožo kot antioksidant v mikronizirani obliki. Prav tako smo predpostavili, da v receptorskem mediju ne bo moč zaznati kozmetično aktivne sestavine. Pri mikroniziranem kvercetinu smo predpostavljali, da ga bo precej ostalo v sloju *stratum corneum* ali da sploh ne bo penetriral v kožo, ali pa ga bomo v globljih plasteh kože zaznali šele po več urah.

Prvi rezultati so nas močno presenetili, saj so bili popolnoma v nasprotju z našimi hipotezami- mikroniziran kvercetin je penetriral precej globlje, hitreje in v večjem obsegu v kožo kot antioksidant v nanosuspenziji. Možni razlogi za dobljene rezultate so nehomogena dispergiranost kvercetina v gelu, neustrezno hidratirana koža (kožo za eksperiment z gelom Poloksamera 407 smo hidratirali 3 dni namesto 24 h, zaradi česar je medij postal moten, prisotni so bili lipidi, beljakovine in odmrle kožne celice), poškodovana koži zaradi ostrih robov delcev kvercetina v mikronizirani obliki in nenatančno odmerjanje gela na kožo. Zaradi

nepričakovanih rezultatov smo se odločili za ponovitev eksperimenta, da bi dobili pričakovane rezultate ali pa dokončno ovrgli naše hipoteze.

Rezultati ponovljenega eksperimenta so pokazali, da je v primeru hidrogela HEC z nanosuspenzijo kvercetina najvišja koncentracija antioksidanta v epidermisu (17,1 $\mu g/cm^2$) nekoliko nižja v *stratum corneum* (13,7 $\mu g/cm^2$) in najnižja v dermisu (8,6 $\mu g/cm^2$). Približno enake rezultate smo dobili pri aplikaciji hidrogela Poloksamera 407 z nanosuspenzijo kvercetina, le da je bila koncentracija kvercetina znatno višja v *stratum corneum*- 25,2 $\mu g/cm^2$ (razlog so lahko npr. močnejši potegi adhezivnih trakov ali neenakomerna razporeditev kvercetina v formulaciji), v epidermisu je znašala 17,2 $\mu g/cm^2$ ter 8,3 $\mu g/cm^2$ v dermisu. Pri hidrogelih z mikroniziranim kvercetinom je bila najvišja koncentracija antioksidanta v *stratum corneum*, kar je pričakovano. Določene koncentracije so bile zelo visoke (20,8 $\mu g/cm^2$ v primeru hidrogela HEC ter 21,9 $\mu g/cm^2$ v primeru hidrogela s Poloksamerom 407), zatorej predpostavljamo, da smo z adhezivnimi trakovi odstranili tudi antioksidant, ki ni penetriral v kožo in je zaradi prevelike velikosti delcev ostal na površini kože. V globlje plasti kože (epidermis in dermis) kvercetin v mikronizirani obliki ne prehaja ali prehaja v zanemarljivih količinah- v primeru hidrogela HEC je bilo zaznanega 1,3 $\mu g/cm^2$ v epidermisu ter 1,9 $\mu g/cm^2$ v dermisu; v primeru hidrogela Poloksamer 407 pa 0,2 $\mu g/cm^2$ v epidermisu in 0 $\mu g/cm^2$ v dermisu. Pri nobenem eksperimentu nismo zaznali antioksidanta v receptorskem mediju, kar kaže, da je tak kozmetični izdelek v skladu z Uredbo o kozmetičnih izdelkih, ki predpisuje, da kozmetično aktivne sestavine ne smejo prehajati skozi kožo.

Rezultati potrjujejo postavljene hipoteze, saj so le-ti v skladu z našimi predpostavkami. Nanokristali kot inovativen dostavni sistem za kozmetično aktivne sestavine so nadgradnja že poznanih kozmetični oblik (npr. hidrogelov) in omogočajo kozmetično aktivnim sestavinam globlje in učinkovitejše prodiranje do tarčnega mesta v koži. Dokazali smo, da kvercetin, kot učinkovit antioksidant, v obliki nanosuspenzije v hidrogelu v relativno velikih koncentracijah prodira v epidermis in dermis, kjer se lahko bori proti oksidativnemu stresu, nevtralizira radikale in tako pomaga pri vzdrževanju učinkovite kožne bariere.

LIST OF USED ABBREVIATIONS

CAS- cosmetic active ingredient

D- Dermis

E- Epidermis

HEC- Hydroxyethylcellulose

NS- Nanosuspension

PI- polydispersion index

SC- *Stratum corneum*

ZP- Zeta potential

1 INTRODUCTION

New techniques and new approaches have been introduced lately in order to improve effectiveness of drugs and cosmetically active ingredients, especially those which possess poor water solubility. Scientists firstly tried to solve the problem with easier techniques such as micronization, adding cosolvents, surfactants or penetration enhancers, usage of fatty solutions instead of those with water, salt formation (1).

However, researches in the field of pharmaceutical nanotechnology presented some innovative solutions for development of formulations with poorly soluble drugs and thus found a way for their incorporation into the market products, as well as making them efficient. Such example is production of nanocrystals.

1.1 Nanocrystals

Nanocrystals as formulation technology are considerably important and new invention in the field of pharmacy, by which scientists have found a way to incorporate poorly water soluble drugs into the products. Nanocrystals are defined as nanoparticles of pure drug without any matrix material with an average size less than 1000 nm (usually in the range between 200 and 500 nm). They could be prepared in different ways in aqueous or non-water media (1). Nanosuspension (NS) is defined as a system, where nanoparticles of an active ingredient either cosmetic or pharmaceutical (<1000 nm) are dispersed in a liquid medium, usually stabilized by using surfactants or polymers (2, 3). By decreasing the size of particles, enlargement of particle surface area and decrease of diffusion layer thickness is achieved. That leads to greater bioactivity, increased saturation solubility and dissolution velocity, which is significantly important for reaching therapeutic concentration on the target site, either in the skin or elsewhere in the body (1). In case of dermal application, the increased concentration gradient between the formulation and the skin results in better penetration of the drug in the skin compared to formulation with larger particles of the same drug (4).

1.1.2 Production of nanocrystals

Nanocrystals can be produced by various methods, which all follow bottom-up, top-down or combination of both approaches. The bottom-up methods are suitable for low water soluble drugs, which are firstly molecularly dissolved in an organic solvent and then dispersed into the surfactants solution, resulting in precipitation of nanocrystals. The top down methods are based on particle decomposition techniques, where micro-sized or larger particles become nano-sized. Top down process includes media milling, microfluidization and high pressure homogenization, while bottom-up technologies include various precipitation-based methods, such as liquid solvent-antisolvent precipitation, precipitation in supercritical fluids and precipitation by solvent-removal. (1)

1.1.2.1 Top-Down Techniques

1.1.2.1.1 Wet ball media milling

Wet ball media milling is one of the most commonly used techniques due to its simplicity and possibility to perform in almost every laboratory; furthermore, it is applicable to all solid materials. Wet ball media milling can be divided in two processes, depending on the amount of energy used. High energy wet milling is procedure where coarse aqueous drug suspension (drug is stabilized with polymers or surfactants to avoid the aggregation) is mixed with media milling beads in a beaker (or jar) by using a roller plate, mixer or the stirrer at really high speeds. The process enables nanocrystal preparation in short time (30-120 min), whereas low energy wet milling procedure demands longer milling time (it can last several days). Since milling is based on constant strong collision between the micro or macrosized particles of drug and beads which causes abrasion, fractures and cleavage and therefore decreases size down to nano range, lower amount of energy leads to less collisions in certain period of time and consequently demands extended timeline. The main drawback of described method is contamination of NS due to the constant abrasion of equipment and/or milling media (beads). But, the techniques have improved and nowadays beads of zirconium alloy, stabilized with yttrium or polystyrene are used, which cause very limited or no contamination (1).

1.1.2.1.2 High pressure homogenization

High pressure homogenization was introduced to the market after the high and low energy wet ball milling process and was initially used for liposome and emulsion preparation. High pressure homogenization can be carried out with three techniques: microfluidizer technology, the piston-gap homogenization in water or the piston-gap homogenization in water-reduced/non-aqueous media. Efficiency of the method depends on the type and power density of device, number of homogenization cycles, drug hardness and initial particle size. In practice it means that softer drugs require less energy for particle size reduction compared to harder ones. The particle size distribution depends on number of cycles (more of them results in better homogenization). Greater equipment power density means easier and quicker achievement of desired particle size (1).

1.1.2.1.3 Microfluidizer technology - insoluble drug formulation technology

The process is based on jet stream homogenizer (microfluidizer), where drug suspension moves through two different chambers at really high speeds and high pressure. Coarse particles of suspension in the first, so called ″Z chamber″, move fast and randomly and therefore collide each other and in the second, so called ″Y chamber″, flow is divided into two new generated flows, where particles are again exposed to collisions, abrasion and cavitation and consequently their size decreases (1). Cavitation is hydrodynamic phenomena, where rapid changes of pressure cause appearance of bubbles in the fluid, inducing them to implode and cause strong shock waves (4). Method does not provide significant size decrease and requires long preparation period and numerous passes through the chambers. Furthermore, it often contains some fraction of microsized particles (1).

1.1.2.2 Bottom-Up Technologies

Instruments for bottom-up technologies are simple, require low energy input and are less expensive if compared with top down systems. Furthermore, they are appropriate for thermolabile drugs since they can be carried out at low temperatures. The main drawback is the use of organic solvents, which have to be removed after the process and therefore the production costs rise. In addition, larger amounts of solvents have to be used in case the drug is poorly soluble in organic solvent. Bottom-up technologies encompass solvent-antisolvent

precipitation, precipitation in supercritical fluids and precipitation by solvent-removal techniques. (1)

1.1.2.2.1 Solvent-antisolvent precipitation

In this method, drug is firstly dissolved in an appropriate solvent (if possible, water miscible solvent, such as ethanol, methanol, acetone, isopropanol is used) and in the next step the obtained solution is mixed with antisolvent (usually water with dissolved stabilizer is used), which has to be miscible with the solvent used. In order to obtain small particles and guarantee quick nucleation, fast stirring is necessary. Some studies have shown that additional external energy input, for example ultrasound, improves the dispersion process and therefore enables production of smaller sized particles. In the last step, dry nanocrystals can be obtained by removing the solvents by lyophilization or spray drying (1).

1.1.2.2.2 Precipitation in supercritical fluids

Supercritical fluids are substances at a temperature and pressure above their critical point, exerting some physicochemical properties of liquids and gasses. They are compressible, behave like gas and have density and typical dissolving power of liquids (1).

The most relevant and commonly used methods for nanocrystal preparation with supercritical fluids are rapid expansion of supercritical solutions and supercritical antisolvent process. In the first method supercritical fluid, such as supercritical carbon dioxide, is used as drug solvent. The drug solution goes rapidly through the aperture or capillary nozzle into the surrounding air. In case of drugs poorly soluble in supercritical fluids the method has been modified - it is called ´rapid expansion of supercritical solution with cosolvent´. In this method (supercritical antisolvent process) the supercritical fluid is used as an antisolvent (1).

1.1.2.2.3 Precipitation by solvent-removal techniques

Methods based on precipitation by solvent removal have been recently introduced to the market and represent new opportunity for nanocrystal preparation. They include

crystallization during freeze drying, spray-freezing into the liquid and nanoprecipitation in microfluidic reactors. In the first technique drug is dissolved in an organic solvent, aqueous solution of cryoprotectant is added and then the sample is immediately freezed and lyophillized. For higher nucleation rate and thus obtaining smaller solvent crystals it is important to increase freezing rate and decrease water content. It leads to reduced interstitial space between crystals and therefore limits the growth of them (1).

1.1.2.3 Combination technologies

Combination technologies were developed for better effectiveness and improvement of individual bottom-up or top-down methods. There are plenty of options to combine two different individual methods and carry them out on higher and more successful level. For example: micro-precipitation followed by high pressure homogenization, pearl milling followed by high pressure homogenization, freeze drying or spray-drying followed by high pressure homogenization etc. In addition to combination of two methods, recently a combination of three methods has been represented, namely precipitation-lyophilization-homogenization method has been introduced (1).

1.2 Hydrogel

Hydrogel is described as a system, made of polymer chains which create 3D network when dispersed in water, which fills the space between them. Polymers should have hydrophilic functional groups in their structure in order to absorb water and should be cross-linked to the certain degree in order to prevent dissolution. There are plenty of methods available for hydrogel cross-linking based on chemical (e.g. polymerization by ionizing radiation, suspension polymerization, solution polymerization and bulk polymerization) or physical interactions (e.g. crystallite formation, entanglements, electrostatic interactions) (5).

Polymers (defined as macromolecules, consisting of different numbers of repeating units), used for hydrogel formation could be natural or synthetic. Latter are usually able to produce more durable structures and thus degrade at slower rate. Examples of synthetic polymers are polyethylenes, polyvinyl chloride, polyamide, aromatic polyesters, phenol-formaldehyde

resins, nylon, Teflon, polysiloxane etc. Natural polymers represent a part of living organisms (for example cellulose polymers can be found in plants) (6).

Hydroxyethylcellulose (HEC)

HEC is natural water-soluble polymer, produced as white powder, usually obtained from wood. It is often used in various cosmetic products in order to thicken the formulation, retain water, provide pseudoplastic characteristics, form films and improve formulation salt tolerance. In addition, it is also known as perfect vehicle for active cosmetic ingredients. HEC-based hydrogels can be easily and quickly prepared as described also in section 3.5 (7,8).

1.3 Antioxidants

Antioxidants are natural or chemically prepared molecules with the main role to prevent oxidative stress in living cells. Oxidative stress is described as unbalanced condition between antioxidants (which defend cells in organism and scavenge free radicals) and free radicals. For example, reactive oxygen species (ROS) and nitrogen reactive species in our body (i.e. superoxide anion radical, hydroxyl radical, singlet oxygen …), caused by various inner conditions or external factors, e.g. smoking, unhealthy food, excessive exposure to sunlight or external toxins, alcohol, stress, water pollutants, pesticides, different diets etc. Free radical is every molecule with unpaired electron in its atomic orbital; therefore, it is unstable and ready to react immediately. In case of excessive amount of free radicals cell damage occurs. Main targets of free radicals are DNA, RNA, proteins, lipids, and carbohydrates. Free radicals also start to harm elastin and collagen fibers in skin layers and consequently wrinkles and loose skin occur. That is why antioxidants are usually added to anti-aging cosmetic products (9).

1.3.1 Quercetin

Quercetin (3,3',4'5,7-pentahydroxyflavone) is a non-water soluble bioflavonoid, found in various natural materials, such as onions, berries, apples, gingko biloba, green tea, American elders etc., giving them characteristic color. It is known for its antioxidant and anti-

inflammatory effects and is widely used in dietary supplements for artherosclerosis, hay fever, heart and vessels diseases, high cholesterol, diabetes, circulation problems, gout, asthma, chronic infections of the prostate and many more (10). Recently some studies dealing with its dermal delivery were conducted, where scientists tested quercetin as antioxidant which could prevent or decrease UVB sunlight skin damage or prevent or minimize destruction of collagen and elastin fibers in the skin layers (10). UVB radiation not only causes premature ageing (by destruction of fibers in the dermis), it also decreases skin defense towards free radicals and oxidative stress. Thus immune system is destroyed or weakened, which can consequently lead to photo carcinogenesis or other diseases. It has been proven that quercetin is effective if applied topically. Researches revealed that nanosized quercetin can penetrate in human or porcine skin at adequate rate and in sufficient amount to provide beneficial effects. It has not been detected to travel transdermally (which is crucial, when incorporated in cosmetic products) (11).

1.4 Skin

Skin represents the largest human organ and provides accurate protection for body. It prevents water evaporation, entrance of microbes, viruses, irritants and allergens, enables the sensation of touch, heat and cold, provides sufficient defense from sunlight, it also represents the shield for inner organs. The skin is composed of three layers, outermost of which in epidermis. It consists of cells called keratinocytes, containing keratin, which are firmly connected with desmosomes. Epidermal cells are slowly moving from basal line to the surface, losing keratin and creating horny layer of dead cells on the skin surface, called *stratum corneum* (the process (desquamation) lasts for approximately 28 days and enables skin to renew). Dermis is located under the epidermis and provides sufficient physical protection- it contains elastic and collagen fibers, made of glycosaminoglycan, which are capable of holding large amount of water and therefore providing skin firmness. Also sweat glands, blood vessels and hair follicles are located in this layer. The inner part of skin is called subcutis (hypodermis) and enables skin to be attached to muscles and bones beneath. Furthermore, it supplies external layers with blood vessels and nerves. Hypodermis contains loose connective tissue, elastin, adipose tissue and big amount of fat which serves as isolation source of skin (12,15).

To carry out dermal experiments pig skin is usually used (example of pig skin is represented in figure 3). It is the most similar to human skin structure and provides most reliable results.

Pig skin has a similar lipid amount of glycosphingolipids and ceramides, collagen and blood vessels composition as human skin, the entire skin thickness is 3,4 mm, whereas the average thickness of human skin is 3 mm. Pigs have approximately 20 hairs per 1 cm^2 (humans have 14-32 (depends on body part)), pig *stratum corneum* measures 21-26 μm (humans have the average thickness of 16 μm), pig epidermis is approximately 66-72 μm thick (human epidermis measures 150 μm).

1.5 Photon correlation spectroscopy and laser Doppler anemometry

Photon correlation spectroscopy (PCS) is widely used method for determination of average particle size and particle size distribution. Colloidal sized particles undergo Brownian motion, which is defined as random movement of colloidal particles in fluid. Smaller particles move more rapidly and larger ones move slower. When the fluid is exposed to laser beam, intensity of scattered light fluctuates depending on particles´ size. Moreover, the correlation lasts for longer if the particles are bigger and it changes quickly in case of smaller particles. To summarize, rapid loss of correlation indicates monodispersal sample and longer presence of correlation shows polydispersal sample (13). The physical stability of NS can be predicted by measuring of their zeta potential (ZP) with laser Doppler anemometry. Based on ZP (which is calculated by particle´s electrophoretic mobility) we can predict stability of a system and possibility of aggregation. In dispersions of particles with ZP of more than 30 mV or less than -30 mV the strong repulsion between dispersed particles is present and thus the stability of the system is good (2, 14).

2 OBJECTIVES

Main purpose of research work is to develop formulation technology, which would improve the dissolution velocity and saturation solubility of quercetin. Since formulation of nanocrystals is relatively novel technology suitable for formulation of water insoluble or poorly water soluble drugs, it seems to be a promising approach for preparation of a topical formulation of the antioxidant quercetin. Firstly, we will prepare four NS differing in stabilizer with wet ball media milling. Afterwards we will characterize the NS by measuring average particle size and particle size distribution (polydispersion index (PI)) with photon correlation spectroscopy and ZP with laser Doppler anemometry. Based on the obtained results we will decide which of the NS is the most suitable for further experiments (considering average particle size, PI and ZP).

We will prepare semisolid formulations with quercetin nanocrystals appropriate for dermal application and investigate how the nanocrystals can improve dermal delivery of quercetin. Therefore, we will incorporate the selected NS of quercetin in two different hydrogels (HEC and Poloxamer 407 hydrogel). The hydrogels with incorporated coarse quercetin particles will be used as reference formulations. Franz cell experiments will be used to test the difference in permeation and penetration of quercetin into different skin layers and into the receptor medium. By this experiment we will also determine the amount of drug accumulated in *stratum corneum* (SC), epidermis (E) and dermis (D) and the amount, which penetrates into the receptor compartment at different time points (2 h, 4 h, 6 h and 8 h after application of formulation on skin sample). Skin will be separated on upper part of epidermis *stratum corneum*, epidermis and dermis and the amount of quercetin in each layer will be determined after extraction by HPLC analysis.

- We assume that quercetin in nanocrystal form will dissolve and diffuse faster, deeper and in higher amount into the skin compared to the coarse quercetin particles.
- We assume that no quercetin will be detected in receptor compartment.
- Coarse quercetin will mostly stay on the skin surface or permeate only into the SC in lower amounts. compared to
- Nanocrystal formulation will also be detected in epidermis and dermis (D, E) after longer exposure time (4 or more hours).

- We do not expect any drug in receptor chamber, after application of the formulation with coarse quercetin particles.
- The antioxidant activity of quercetin nanocrystals is, due the better skin penetration, much higher compared to the antioxidant potential of coarse quercetin particles.

3 MATERIALS AND METHODS

3.1 Materials

The following materials were used in this study: active cosmetic ingredient was quercetin (Sigma Life science, St. Louise, USA), stabilizers were Tween 80, polysorbate 80 (Galeno srl, Comeana, Italy) and poloxamer 188. Hydrogels were made of hydroxyethylcellulose (Galeno srl, Comeana, Italy), glycerol (Galeno, srl, Comeana, Italy) and poloxamer 407 (Lutrol F 127, BASF The chemical company, Ludwigshafen,Germany).

3.2 Equipment

Laboratory equipment:

- Beakers, flasks
- Injections with measuring scale
- Microcentrifuges
- Mortar and pestle
- Test tubes, test tube rack
- Pipettes
- Scalpel, scissors, glass rod, spatulas

Machines:

- Balance: Kern& Sohn GmbH, ser. No.: WB12AA0278
- Homogenizator: Ultra Turax T25 Basic IKA Werke
- Wet ball milling device: Disruptor genie, Scientific industries
- Zeta Sizer and PCS device: Malvern instruments, Nano ZS
- Sonificator: Soniprep 150 plus, MSE

3.3 Preparation of nanosuspensions

We prepared 3 % and 5 % (w/w) quercetin NS with Poloxamer 188 as stabilizer,composition of each is presented in table I. For 3% quercetin NS we firstly dissolved the stabilizer in water and added the solution into 10 mL beaker, where antioxidant quercetin has been previously weighted. After 1 min of homogenization by rotor-stator homogenizer (9.500 rpm), the obtained suspension was divided in four aliquots in 1 mL vials and 0.4 g of silibeads, which aid to decrease the size of particles during the milling process, were added into each vial. Afterwards WBW was performed as follows: vials were put in the wet milling device shown in the figure 1. During 90 min of milling the NS was characterized every 15 min. We continued with preparation of 5 % quercetin NS (prepared according to the same method as the 3 % NS).

Figure 1: Wet ball media milling device

Table I: Composition of 3 % and 5 % (w/w) quercetin nanosuspension with poloxamer 188 as stabilizer

	Quercetin [mg]	Poloxamer 188 [mg]	Water [mg]	Total amount [mg]
NS Que3% Pol	30	10	960	1000
	150	50	4800	5000
NS Que5% Pol	50	10	940	1000
	250	50	4700	5000

The following 3 % and 5 % quercetin NS were prepared with Tween 80 (Polisorbat 80) instead of Poloxamer 188 (formulation is reported in the table II), because we wanted to compare two stabilizers and decide for the more effective one. The procedure was the same as described for Poloxamer 188 NS.

Table II: Composition of 3 % and 5 % (w/w) quercetine nanosuspension with Tween 80 as stabilizer

	Quercetin [mg]	Tween 80 [mg]	Water [mg]	Total amount [mg]
NS Que3%Tween	30	10	960	1000
	150	50	4800	5000
NS Que5%Tween	50	10	940	1000
	250	50	4700	5000

To obtain the sufficient amount of NS for incorporation in hydrogels, we repeated the procedure as described before (for 3 % quercetin NS with Tween 80). We prepared 12 parallels, milled them for 90 minutes, combined them in one beaker and poured the obtained NS through the sieve to get rid of the silibeads. We used this NS for further permeation experiments with Franz cells. Firstly, we prepared Poloxamer 407 and HEC hydrogels as described in section 3.5, then we incorporated NS in hydrogels and finally we have prepared the reference formulations i.e. the hydrogels with coarse quercetin particles.

3.4 Characterization of nanosuspensions

NS were characterized every 15min. After 30 min, 45 min, 60 min, 75 min and 90 min of wet ball media milling we carried out the measurement for average particle size, PI and ZP. We sipped the supernatant from the vial with a syringe, added approximately 3 mL of water (the mixture in the syringe should be nearly transparent) and then transferred it into a vial or capillary cell for PCS or ZP analysis s. The vial should be dry on the outside in order not to damage the machine. Furthermore, there should be no bubbles inside the sample, because the machine does not differ between our quercetin nanosized particles and other nanosized structures in the vial. The vial for zeta potential measurements should be filled above the minimum and under the maximum level marked on the vial. Additionally, it is very important to fill it properly i.e. lean it on one side and carefully transfer the solution in it with the injection without any bubbles. The vials were washed with water and ethanol after each measurement.

3.5 Preparation of hydrogels

We prepared two different hydrogels. HEC hydrogel was composed from 2.5 % (w/w) HEC, 10 % (w/w) glycerin and 87.5 % (w/w) water. To prepare 100 g of hydrogel, 2.5 g of HEC was weighted and combined with 10 g of glycerin and put on the vortex to sufficiently mix. Afterwards, 87.5 g of boiled and cooled down water was slowly added during the constant stirring. Then hydrogel was left covered for about an hour to swell and obtain its viscose structure. Poloxamer 407 gel was prepared by simple mixing of 20 % (w/w) of Poloxamer 407 and 80 % (w/w) of boiled and cooled down water during constant stirring. The amount of prepared hydrogel was 100 g, thus we weighted 20 g of Poloxamer 407 and 80 g of water.

3.6 Preparation of hydrogel with quercetin nanosuspension

Hydrogels (HEC and Poloxamer 407) with NS were prepared simply by sufficiently mixing the NS into the hydrogel with pestle after the hydrogels were already produced. Since we wanted to prepare similar formulation to dr. Müller´s in his experiment (2), the final hydrogel

should contain 15 % of quercetin NS, therefore we mixed 600 mg of 3 % quercetin NS and 3400 mg of Poloxamer 407 or HEC hydrogel (total amount of 4000 mg).

3.7 Preparation of quercetin hydrogel with coarse quercetin particles

We prepared a reference hydrogel with coarse quercetin particles with the same drug loading as hydrogels with NS. Therefore, we prepared 100 g hydrogel and added 18 mg of quercetin powder and mixed well. The reference formulation thus contained 0.045 % (w/w) of quercetin.

3.8 Permeation experiments using Franz cells

Franz cell is vertical static cell, used for determination of permeability of compounds through synthetic membrane or organic tissues, to determine the compound flux (permeation rate across the membrane) and to detect concentration of the drug in the receptor chamber (16). It consists of a donor compartment, where the drug or formulation is applied, and a receiver chamber, where penetrated drug is collected. The skin is inserted between both compartments, in the way that *stratum corneum* faces the donor compartment and the dermis faces the receptor compartment of Franz diffusion cell. The entire set up is clamped and fastened with parafilm. A magnetic stirring bar is inserted in the receptor compartment to assure sink conditions with constant mixing, while circulating water jacket maintains constant physiological temperature (37 °C). An example of Franz cell experiment is shown in figure 2.

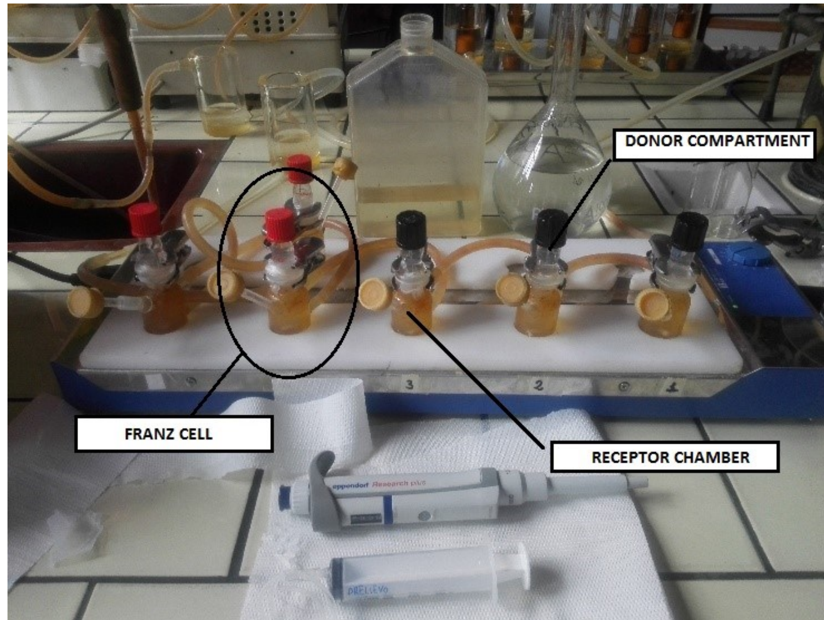

Figure 2: Franz cells with marked receptor and donor compartment

For the experiment we cut 6 round pieces of newborn pig skin (full thickness with hair) A skin samples are shown in figure 3., Then we filled the receptor compartment with saline, put skin *(stratum corneum* facing the donor compartment) over the receptor compartment and covered it with donor compartment. We fastened donor and receptor chamber together with paraffin tape and a clamp. Then we filled the donor compartment with saline and left it over the night so the skin could be sufficiently hydrated.

Figure 3: Pig skin used for Franz cell experiment

Next day we began with the experiment. The saline was firstly removed from the donor compartment and substituted with 200 µL of HEC hydrogel containing 3 % quercetin NS with Tween 80. Every 2 h (2 h, 4 h, 6 h, 8 h) we removed the receptor medium from the receptor compartment and replaced it with the fresh one. The receptor medium with the penetrated drug (quercetin) was stored in the vials in the freezer (-20 °C) till measurements were carried out. We performed the experiment 3 times. Firstly, we used 6 Franz cells with 3 % (w/w) quercetin NS with Tween 80 in HEC hydrogel, secondly 6 Franz cells with 3 % (w/w) quercetin NS with Tween 80 in Poloxamer 407 hydrogel and thirdly, 3 Franz cells with coarse quercetin in HEC hydrogel and 3 Franz cells with coarse quercetin in Poloxamer 407 hydrogel.

At the end of each experiment we removed the skin from the Franz cells and stored it rolled up in an aluminium foil in a freezer (-20 °C).

After lyophilization we scratched the white powder from the walls and bottoms of vials, added 2 mL of methanol in each vial, mixed well with a vortex mixer for approximately 30 s, transferred the mixtures into HPLC vials and stored them in a freezer (-20 °C) till the measurements.

3.9 Analysis of quercetin in pig skin layers

After the permeation experiment, we separated the pig skin on individual layers in order to determine amount of quercetin in each of them. Firstly, we removed the *stratum corneum* with adhesive tapes. We firmly pressed the tape onto the skin surface and pulled it off with one fluent stroke. We used 3 different tapes and performed 3 pull-offs with each. We cut the tapes into small pieces and put them into the vial loaded with 3 mL of methanol.

Secondly, we separated the dermis from epidermis by using surgical scalpel and put the dermis into the vial loaded with 2 mL of methanol. Finally, we cut the epidermis into small pieces and put them into a vial loaded with 2 mL of methanol. We sonicated the samples to accelerate quercetin extraction and dissolution in methanol. Sonication lasted 120 s (on/off sonication with 2 60 s cycles). The samples without tissue parts were transferred into HPLC vials and put into a freezer (-20 °C) until the measurements.

3.10 HPLC measurements

The quantity of quercetin in different skin layers as well as in receptor compartment was determined by HPLC analysis. All samples were put into the HPLC vials prior analysis with HPLC machine. We analyzed the following samples: receptor media of HEC hydrogel with 3 % (w/w) quercetin NS at four different time points, receptor media of Poloxamer 407 hydrogel with 3 % (w/w) quercetin NS at four different time points, receptor media of HEC hydrogel with coarse quercetin particles at four different time points, receptor media of Poloxamer 407 hydrogel with coarse quercetin particles at four different time points and samples of s*tratum corneum*, epidermis and dermis of all experiments.

4 RESULTS AND DISCUSSION

4.1 Nanosuspension measurements

In order to choose the most optimal NS for further permeation experiment we prepared 4 different NS, differing in stabilizer used (Poloxamer 188 or Tween 80) and concentration of quercetin (3 % or 5 % (w/w)). Obtained results are represented in tables III, IV, V and VI.

Table III: Representation of measurements for all parallels of 3 % (w/w) quercetin nanosuspensions stabilized with poloxamer 188 - average size, zeta potential and polydispersity index

Measurement / Milling time [min]	**15**	**30**	**45**	**60**	**75**	**90**
Average particle size [nm]	794.8 +/- 36	661.4 +/- 14.9	758.6 +/- 25.9	769.8 +/- 30.7	610.7 +/- 3.1	858.2 +/- 22.7
	697.9 +/- 11.6	632.5 +/- 10.7	607.6 +/- 9.2	918.4 +/- 12.3	842.6 +/- 16.9	542.8+/- 4.6
	845.2 +/- 29.6	1147.0 +/- 13.6	611.1 +/- 10.5	873.5 +/- 31.4	574.1 +/- 15.2	604.0 +/- 2.8
	1039.0 +/- 41.8	990.2 +/- 35.9	994.4 +/- 26.5	628.1 +/- 23.2	539.7 +/- 11.4	763.3 +/- 25.5
Average [nm]	**844.2**	**857.8**	**742.9**	**797.5**	**641.8**	**692.1**
PI	0.113 +/- 0.077	0.146 +/- 0.002	0.217 +/- 0.055	0.285 +/- 0.029	0.144 +/- 0.066	0.315 +/- 0.046
	0.178 +/- 0.051	0.045 +/- 0.001	0.203+/- 0.016	0.276 +/- 0032	0.291 +/- 0.044	0.169 +/- 0.017
	0.120 +/- 0.118	0.309 +/- 0.053	0.149 +/- 0.054	0.253 +/- 0.048	0.072 +/- 0.081	0.106 +/- 0.076
	0.328 +/- 0.078	0.331 +/- 0.072	0.291 +/- 0.062	0.104 +/- 0.036	0.161 +/- 0.021	0.347 +/- 0.018
Average	**0.184**	**0.207**	**0.215**	**0.230**	**0.167**	**0.234**
Zeta Potential [mV]	-33.6	-39.1	-39.2	-38.5	-39.6	-39.3
	-34.9	-37.7	-36.8	-39.5	-38.9	-37.9
	-39.3	-39.8	-39.2	-39.8	-36.8	-37.5
	-38.2	-39.8	-40.6	-39.6	-36.5	-39.0
Average [mV]	**-36,5**	**-39,1**	**-39,0**	**-39,4**	**-38,0**	**-38,4**

Table IV: Representation of measurements for all parallels of 5 % (w/w) quercetin nanosuspensions stabilized with poloxamer 188 - average size, zeta potential and polydispersity index

Measurement / time [min]	**15**	**30**	**45**	**60**	**75**	**90**
Average size [nm]	575.8 +/- 13.0	637.3 +/- 24.2	614.0 +/- 7.9	644.9 +/- 18.3	692.4 +/- 8.8	601.3 +/- 19.8
	539.4 +/- 3.1	854.0 +/- 27-9	883.6 +/- 26.1	649.6 +/- 11.7	856.9 +/- 15.5	507.5 +/- 17.9
	692.7 +/- 25.6	841.4 +/- 21.0	646.4 +/- 11.6	1171.0 +/- 46.5	690.6 +/- 22.5	753.7 +/- 25.0
	632.6 +/- 40.1	664.2 +/- 22.2	715.2 +/- 38.3	764.7 +/- 21.2	622.2 +/- 17.7	803.7 +/- 28.0
Average of parallels	**610.1**	**749.2**	**714.8**	**807.6**	**715.5**	**666.6**
PI (polidispersion index)	0.167 +/- 0.036	0.185 +/- 0.108	0.210 +/- 0.059	0.103 +/- 0.046	0.280 +/- 0.026	0.264 +/- 0.038
	0.183 +/- 0.040	0.211 +/- 0.029	0.314 +/- 0.049	0.126 +/- 0.107	0.307 +/- 0.060	0.071 +/- 0.010
	0.223 +/- 0.021	0.343 +/- 0.060	0.244 +/- 0.064	0.333 +/- 0.031	0.092 +/- 0.026	0.196 +/- 0.060
	0.143 +/- 0.101	0.112 +/- 0.087	0.199 +/- 0.022	0.150 +/- 0.057	0.267 +/- 0.036	0.211 +/- 0.020
Average of parallels	**0.179**	**0.213**	**0.241**	**0.178**	**0.237**	**0.188**
Potential zeta [mV]	-41.8	-36.6	-39.1	-37.6	-37.7	-40.4
	-39.2	-37.3	-38.9	-38.8	-40.4	-39.3
	-40.2	-39.2	-39.0	-36.9	-36.1	-35.6
	-41.0	-37.9	-37.5	-37.4	-36.4	-36.8
Average of parallels	**-40.6**	**-37.8**	**-38.6**	**-37.7**	**-37.7**	**-38.2**

Table 1: Representation of measurements for all parallels of 3% Tween 80 nanosuspensions- average size, potential zeta, polydispersion index

Measurement / time [min]	**15**	**30**	**45**	**60**	**75**	**90**
Average size [nm]	429.6 +/- 4.6	398.0 +/- 3.7	378.1 +/- 7.5	310.8 +/- 6.1	303.7 +/- 5.2	334.5 +/- 12.2
	440.4 +/- 11.0	579.6 +/- 15.7	370.2 +/- 5.0	567.1 +/- 3.8	316.3 +/- 9.1	306.5 +/- 8.0
	459.9 +/- 6.7	412.2 +/- 21.9	381.1 +/- 2.4	345.4 +/- 4.8	309.2 +/- 4.8	314.9 +/- 11.7
	677.1 +/-	413.7 +/-	369.1 +/-	334.5 +/-	313.6 +/-	309.2 +/-

	21.1	8.3	9.0	12.3	0.3	2.1
Average of parallels [nm]	**501.8**	**450.9**	**374.6**	**389.5**	**310.7**	**316.3**
PI (polidispersion index)	0.274 +/- 0.027	0.179 +/- 0.046	0.176 +/- 0.032	0.192 +/- 0.021	0.175 +/- 0.047	0.170 +/- 0.033
	0.258 +/- 0.016	0.405 +/- 0.021	0.187 +/- 0.011	0.324 +/- 0.015	0.162 +/- 0.052	0.151 +/- 0.030
	0.302 +/- 0.073	0.260 +/- 0.026	0.232 +/- 0.024	0.167 +/- 0.011	0.124 +/- 0.038	0.136 +/- 0.015
	0.446 +/- 0.056	0.264 +/- 0.014	0.216 +/- 0.024	0.159 +/- 0.010	0.176 +/- 0.004	0.177 +/- 0.054
Average of parallels	**0.320**	**0.277**	**0.202**	**0.211**	**0.159**	**0.159**
Potential zeta [mV]	-40.9	-38.6	-38.7	-37.4	-38.0	-38.2
	-40.5	-37.8	-38.1	-37.3	-38.5	-38.2
	-40.2	-37.3	-379	-36.9	-37.1	-38.6
	-38.5	-37.9	-38.7	-40.1	-37.6	-37.7
Average of parallels [mV]	**-40.0**	**-37.9**	**-38.4**	**-37.9**	**-37.8**	**-38.2**

Table 2: Representation of measurements for all parallels of 5% Tween 80 nanosuspensions- average size, potential zeta, polydispersion index

Measurement / time [min]	**15**	**30**	**45**	**60**	**75**	**90**
Average size [nm]	4984 +/- 2.2	688.1 +/- 7.7	405.5 +/- 8.6	659.9 +/- 22.7	496.0 +/- 8.0	623.8 +/- 33.8
	345.7 +/- 16.0	414.4 +/- 5.5	398.2 +/- 2.0	448.7 +/- 7.6	723.3 +/- 7.0	615.8 +/- 9.0
	498.6 +/- 3.7	642.2 +/- 8.1	462.0 +/- 21.1	411.6 +/- 13.7	604.6 +/- 13.0	360.1+/- 1.7
	569.6 +/- 17.7	438.4 +/- 5.0	418.2 +/- 9.6	469.7 +/- 6.0	447.2 +/- 14.5	557.2 +/- 2.3
Average of parallels [nm]	**478.1**	**545.8**	**420.97**	**497.5**	**567.8**	**539.2**
PI (polidispersion index)	0.238 +/- 0.029	0.324 +/- 0.038	0.071 +/- 0.051	0.282 +/- 0.013	0.086 +/- 0.081	0.217 +/- 0.038
	0.337 +/- 0.092	0.222 +/- 0.026	0.167 +/- 0.040	0.191 +/- 0.039	0.221 +/- 0.024	0.251 +/- 0.065
	0.276 +/-	0.300 +/-	0.255 +/-	0.188 +/-	0.271 +/-	0.106 +/-

	0.051	0.006	0.021	0.088	0.008	0.006
	0.311 +/- 0.013	0.194 +/- 0.032	0.168 +/- 0.019	0.198 +/- 0.014	0.155 +/- 0.020	0.288 +/- 0.011
Average of parallels	**0.293**	**0.260**	**0.165**	**0.215**	**0.182**	**0.215**
Potential zeta [mV]	-38.7	-38.4	-39.8	-40.6	-40.0	-40.5
	-38.6	-38.5	-38.7	-39.1	-39.3	-39.5
	-38.4	-38.3	-39.0	-40.0	-39.1	-39.8
	-39.6	-39.0	-38.8	-39.3	-39.7	-40.2
Average of parallels [mV]	**-38.8**	**-38.6**	**-39.1**	**-39.8**	**-39.5**	**-40.0**

After the characterization of all prepared NS we decided that the one with 3 % (w/w) quercetin stabilized with Tween 80 has the most optimal properties for incorporation in hydrogels. It showed the smallest average particle size and the lowest PI, as well as continuous decrease in particle size during the milling process. We did not observe any aggregation, which most probably occurred in case of NS with 5 % quercetin stabilized with Poloxamer 188. In this formulation the average particle size fluctuated during the milling process and it was even bigger at the end of milling (666.6 nm at the final time point i.e. 90 min) compared to the average particle size obtained after 15 min of milling (610.1 nm). Furthermore, PI of 3 % (w/w) quercetin NS stabilized with Tween 80 decreased during milling process; that means particles became more homogeneous in size, due to abrasion, collision and hitting during the milling. From the results we can conclude that Poloxamer 188 is not suitable stabilizer for quercetin NS. Even though PI values were within the desired range (under 0.250), the particle size did not decrease with increased milling time. There were ups and downs in average particle size, most probably due to particle aggregation. NS with 5 % (w/w) quercetin stabilized with Tween 80 did not reveal expected results as well. The PI was under 0.200 after 45 min of milling, but the particles size raised from the first time point till the end of milling process. A possible explanation of this behavior could be that the concentration of quercetin was too high and added amount of Tween 80 could not provide sufficient stabilization of particles.

After choosing the most optimal NS we repeated the entire process once more and obtain larger amount of NS for further experiments. Characteristics of this one are represented in table VII.

Table VII: Average particle size, zeta potential and polydispersity index of 3 % (w/w) quercetin nanosuspension stabilized with Tween 80 used in permeation experiments

Average particle size [nm]	517.1 +/- 13.1
Zeta potential [mV]	-40.1
PI	0.120 +/- 0.008

Photon correlation spectrometry was used for average particle size and particle size distribution analysis. Average size of particles is a very important parameter since it affects physical stability, dissolution rate and saturation solubility. Smaller the particles are, larger is their surface and consequently it enables the drug to become bioavailable and reactive. Average size of the particles should be less than 1000 nm, other way we cannot consider the mixture as nano delivery system. Results shown in table VIII indicate that longer wet milling process results in smaller average particle diameter.

ZP is indicator for dispersion stability since the presence of a surface charge on nanoparticle prevents their aggregation. Ideal ZP value for electrostatically stabilized NS is outside the range from -30 mV and +30 mV, inside this range the system is considered as unstable. For NS, stabilized with steric stabilizer, the perfect values are less than -20 mV or more than +20 mV (2). Since ZP of quercetin NS stabilized with Tween 80 was less than -30 mV it indicates good particle stabilization (results are demonstrated in table VII).

After incorporating the NS into the HEC and Poloxamer 407 hydrogels we performed measurements for average size, polydispersal index and zeta potential (results are represented in table VIII). Measurements were performed identically to those with nanosuspensions.

Table VIII: Average particle size, polydispersity index, and zeta potential of HEC and poloxamer 407 hydrogels with incorporated quercetin NS

Type of measurement/ type of hydro gel	**HEC hydrogel + nanosuspension**	**Poloxamer 407 hydrogel + nanosuspension**
Average particle size [nm]	567.9 ± 6.1	368.9 ± 9.1
PI	0.181 ± 0.011	0.198
ZP [mV]	-31.0	-25.3

4.2 Penetration of quercetin in and through the skin

Results obtained after the first penetration experiments revealed some unexpected data i.e. quercetin in form of coarse quercetin particles in HEC hydrogel penetrated in the skin faster and in higher amount compared to quercetin in nanocrystals in HEC hydrogel. The amount of quercetin accumulated in *stratum corneum* after application of coarse quercetin suspension was almost three times bigger than that obtained after application of HEC hydrogel loaded with quercetin NS. We decided to repeat the experiment in order to confirm the obtained data or to find the reason for strange results in the first experiment. The following are some possible explanations for the obtained results:

- The coarse quercetin particles were not, due to big particle size, homogeneously dispersed in hydrogel and the dose of quercetin applied on the skin was thus higher. The skin specimen used for the experiment lost its integrity due to too long hydratation process. According to method, hydratation should last for one day (24 h), however, we left the Franz cells over the weekend (more than 72 h). In addition, quercetin in receptor compartment could not be correctly detected due to the presence of lipids, proteins and skin cells in the media (receptor department was cloudy).
- The coarse quercetin particles could have damaged the pig skin, due to particle sharpness, and thus enter the skin in higher concentration.
- The pig skin used for the experiment could have been damaged.
- Due to difficult application of the sample on the skin (very viscous formulation) part of the sample could stay on the walls of donor compartment and did not reach the skin, therefore there could be variations in quercetin concentration.
- With the first tape stripes we took some quercetin particles from the skin surface and not only the quercetin, which penetrated in *stratum corneum.*

Following results show how quercetin penetrated in and through the skin in second experiment (tables IX and X).

Table IX: Concentration of quercetione in different skin layers. SC= stratum corneum, EP= epidermis, DE= dermis.

Concentration	HEC hydrogel with	HEC hydrogel withcoarse	Poloxamer 407 hydrogel with	Poloxamer 407 hydrogel with

[µg/cm^2]	nanosuspension	quercetin	nanosuspension	coarse quercetin
SC	13,7	20,8	25,2	21,9
EP	17,1	1,3	17,2	0,2
DE	8,6	1,9	8,3	0

Table X: Results of Franz cell experiment- Percentage of quercetin dose in different skin layers and in receptor compartment. SC= stratum corneum, EP= epidermis, DE= dermis, RC= receptor chamber.

	HEC hydrogel with nanosuspension	HEC hydrogel withcoarse quercetin	Poloxamer 407 hydrogel with nanosuspension	Poloxamer 407 hydrogel with coarse quercetin
SC % DV	6,1	11,4	7,3	2,15
EP% DV	2,0	0,8	1,7	0,1
DE% DV	1,3	0,6	3,0	0
RC%DV	0	0	0	0

The first, quick look at the obtained values reveals the expected results i.e. higher amount of quercetin in outer skin layers, lower concentration in deeper ones and no cosmetic ingredient detected in receptor medium. However, when we looked closer and more precisely, we observed some deviations, especially when comparing concentrations of quercetin permeated from NS formulation and formulation with coarse quercetin particles.

Table IX shows that the highest amount of quercetin from HEC hydrogel loaded with quercetin NS penetrated into epidermis (17.1 µg/cm^2) and noticeable concentration was also present in *stratum corneum* (13.7 µg/cm^2). Similar results were obtained with quercetin NS in Poloxamer 407 hydrogel, even though there was higher concentration in dermis (25.2 µg/cm^2), the concentration in epidermis was almost the same (17.2 µg/cm^2) as concentration of NS in HEC hydrogel. These results clearly show how the cosmetic ingredient in form of nanosized particles penetrates deeper and in higher amount in the skin compared to coarse particles. The concentrations of quercetin which penetrated into dermis from NS loaded in HEC and Poloxamer 407 hydrogel were 8.6 µg/cm^2 and 8.3 µg/cm^2, respectively, which is

undoubted much higher than the concentration which penetrated from formulations with micronized drug (1.9 μg/cm^2 in case of HEC hydrogel and 0 μg/cm^2 in case of Poloxamer 407 hydrogel). No quercetin was detected in receptor chamber after application of formulation with coarse quercetin particles, therefore the formulation complies with the law for cosmetic products. Quercetin applied in form of coarse quercetin in HEC and Poloxamer 407 hydrogels reached pretty high concentrations in *stratum corneum* i.e. 20.8 μg/cm^2 and 21.9 μg/cm^2 respectively. Those concentrations (20.8 μg/cm^2 and 21.9 μg/cm^2) are much higher compared to quercetin concentration in *stratum corneum* after application of quercetin NS loaded HEC hydrogel (13.7 μg/cm^2). The reasonable explanation could be accumulation of ingredient on the skin surface, and not really in *stratum corneum*, which we pulled off with stripes. The reason could be also inhomogenously dispersed quercetin particles in hydrogel. Concentrations in outer most skin layer are not alarming, since there are many explanations for obtaining them. Bigger concern would raise same conditions in deeper skin layers (epidermis, dermis), that would reveal huge mistakes in our method, since it is not logical for coarse drug to penetrate deeper and in greater extent than nano sized drug. Despite some unexpected deviations in the obtained results, they generally prove that quercetin NS are better and more efficient delivery system compared to micro sized particles of cosmetic ingredient.

The first part of table IX (quercetin NS in HEC hydrogel) shows that the higher percentage of quercetin has penetrated in *stratum corneum* (6.1 %), less in epidermis (2.0 %) and the lowest percentage of active cosmetic ingredient reached the dermis (1.3 %). Results show that quercetin penetrates into the top skin layer (SC) and mostly stays there, even though some has been detected also in epidermis and dermis. These findings are very important, since collagen and elastin fibers are located there and can be thus protected by the flavonoid against UV induced damage. The presence of antioxidant is important also in *stratum corneum*, where it can protect the outer skin cells against sun rays and negative environment influences. The important information is also that no quercetin reached the receptor chamber, thus the formulations comply with the current law for cosmetic products.

The following part of table IX presents the results obtained with formulation of coarse quercetin particles in HEC hydrogel. There was surprisingly high percentage of quercetin in *stratum corneum* (11.4 %) which is significantly higher than in the case of quercetin NS loaded hydrogel, where only 6.1 % was detected in *stratum corneum*. The reason for the

obtained results could be the same as pointed out before. The percentage of quercetin, which permeated from formulations loaded with coarse quercetin particles, in epidermis was only 0.8 % and 0.6 % in dermis. These results have confirmed that ingredient in form of coarse particles cannot penetrate deep in the skin due to its size and shape.

The third part of table IX represents the information about quercetin permeation from quercetin NS in Poloxamer 407 hydrogel. The amount of quercetin in *stratum corneum* was pretty high (7.3 %), a little higher than in *stratum corneum* in case of quercetin NS in HEC hydrogel. As already discussed above, the reason could be inhomogeneous distribution of quercetin in formulation, therefore we could have sampled the part with higher concentration. The reason for higher amount could also be stronger pull-offs with tape, compared to those in case of hydrogels with NS. The percentages of quercetin in epidermis (1.7 %) and dermis (3.0 %) were very similar to those achieved with HEC hydrogel formulation.

The fourth part shows that active cosmetic ingredient penetrated only in *stratum corneum* (2.2 %) and epidermis (0.1 %) in case of coarse quercetin particles dispersed in Poloxamer 407 hydrogel, and none of it reached the dermis or receptor chamber.

As far as quercetin NS are concerned, results are reasonable. The main finding is that quercetin nanocrystals did not permeate through skin. Indeed, this fact allows us to use the quercetin NS in cosmetic products, since according to the current European law, transdermal penetration of dermally applied cosmetic substances is prohibited. The active cosmetic ingredient should remain in *stratum corneum*, epidermis and dermis. The highest concentration of quercetin was detected in *stratum corneum* and smaller amounts in epidermis and dermis. Since antioxidants protect the skin from the effects of UV radiation and prevent degradation of collagen and elastic fibers, which are important component of dermis, it is important that quercetin reaches dermis. Our formulation is in this aspect very promising.

6 CONCLUSIONS

In this research we have prepared and characterized quercetin nanocrystals and tested them as a formulation for dermal quercetin delivery. The aim was to highlight the advantages of new delivery system in cosmetics for easy and effective delivery of antioxidants in the skin. Since nano sizing is one of the most innovative approaches to improve topical delivery of poorly water soluble active ingredients incorporated in cosmetic products, we have confirmed some clear advantages of such formulations in comparison to those containing coarse quercetin particles. Here are some of the most important findings:

-The most promising quercetin NS, regarding the average particle size, PI and ZP, was shown to be 3 % (w/w) quercetin NS stabilized with Tween 80.

-Poloxamer 188 was shown not to be suitable stabilizer for quercetin NS, since little aggregation occurred.

-Furthermore, 5 % (w/w) quercetin NS stabilized with Tween 80 was not suitable because some extent of aggregation occurred.

-After incorporation of NS into Poloxamer 407 and HEC hydrogel, we discovered that both formulations behave similarly i.e., particles in HEC hydrogel were more stable than in Poloxamer 407 hydrogel, since ZP was close to -30 mv. The experiments showed that quercetin in form of NS incorporated in HEC and Poloxamer 407 hydrogel penetrates in epidermis and dermis in almost same amounts and that quercetin in NS in Poloxamer 407 hydrogel penetrated into the *stratum corneum* in higher amount compared to quercetin NS in HEC hydrogel.

-If the main target of quercetin is the *stratum corneum,* we should use Poloxamer hydrogel and if the target is epidermis or dermis, we can use whichever we want.

-As we presumed, the majority of coarse antioxidant stays in *stratum corneum* or even on the skin surface and really small amount or nothing at all enters the epidermis and dermis.

-We hypothesized that the quercetin penetration in the deeper skin layers could happen after a long exposure, but the experiment has shown that it did not permeate into the skin in time of more than 8 h.

-The intact skin barrier does not allow nanoparticles to diffuse into the skin. Moreover, quercetin is a lipophilic molecule which can penetrate, but tends to accumulate in the skin

layers. That is very important fact, since the cosmetic law prohibits any cosmetic ingredient to penetrate through full skin thickness and to enter the blood system. To sum up, we can claim that quercetin NS is an effective innovative delivery system and can be used in various cosmetic products. What is more, the antioxidant does not penetrate through the skin *in vitro*, indicating that it will most probably not enter the blood stream *in vivo* and cause any systemic side effects.

7 LITERATURE

1. Lai F., Schlich M., Pireddu R., Corrias F., Fadda M. A., Sinico C.: Production of Nanosuspensions as a Tool to Improve Drug Bioavailability: Focus on Topical Delivery. Current Pharmaceutical Design. 21. 2015. 6089-6103.

2. Patel V.R., Agrawal Y. K.: Nanosuspension: An approach to enhance solubility of drugs. Journal of advanced Pharmaceutical Technology and Research. April-June 2011. 2(2). 81-87.

3. Fonda N.: study of redispersibility of nanosuspension with poorly soluble drug. Final thesis. 2012. Ljubljana: Faculty of pharmacy.

4. Shegokar R., Müller H. R.: Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives. International Journal of Pharmaceutics. 399. 2010. 129-139.

5. Enas M. A.: Hydrogel: Preparation, characterization, and applications. Journal of Advanced Research. 6 (2). March 2015. 105-121.

6. http://study.com/academy/lesson/natural-polymers-definition-types-examples.html (Accessed on 6.4.2016)

7. http://www.dow.com/dowwolff/en/industrial_solutions/polymers/hydroxyethylcellulose/ (Accessed on 6.4.2016)

8. http://www.ingredientstodiefor.com/item.php?item_id=1333 (Accessed on 6.4.2016)

9. Lobo V, Patil A., Phatak A., Chandra N.: Free radicals, antioxidant and functional foods. Pharmacognosy review. 4(8). July- December 2010. 118-126.

10. http://www.webmd.com/vitamins-supplements/ingredientmono-294-quercetin.aspx?activeingredientid=294& (Accessed on 16.6.2017)

11. Testa Moura de Carvalho Vicentini F., Geogretti S. R., Lopes Badra Bentley M. V., Vieira Fonseca M. J.: Assessment of in vitro methodologies to determine topical and transdermal delivery of flavonoid quercetin. Brazilian Journal of Pharmaceutical Sciences. 45. June 2009. 357-364.

12. http://www.britannica.com/science/human-skin (Accessed on 5.4.2016)

13. Tscharnuter W.: Photon Correlation Spectroscopy in Particle Sizing. Encyclopedia of Analytical Chemistry R.A. Meyers (Ed). 2000. pages: 5469-5485.

14. Božič V.: Preparation and characterization of dexamethsone nanosuspensions for dermal application. Master thesis. 2016. Ljubljana: Faculty of pharmacy.

15. Netzlaff F., Lehr C. M., Schaefer U.F.: The human epidermis models Episkin, SkinEthic and EpiDerm: An evaluation of morphology and their suitability for testing phottoxity, irritancy, corrosivity, and substance transport. European Journal of Pharmaceutics and Biopharmaceutics 60. March 2005. 167-178.

16. http://permegear.com/wp-content/uploads/2015/08/primer.pdf (Accessed on 12.4.2016)