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Antioxidant delivery systems for cosmetic application

Microencapsulation of rosmarinic acid by spray drying and single emulsion solvent evaporation

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Abstract

Antioxidants are molecules capable of oxidizing themselves instead or before other molecules. For cosmetic application, antioxidants may be used to retard the skin ageing process caused by oxidative stress. In addition, this ingredient may also be used as preservative avoiding the rancidification of the product or protecting other sensitive ingredients from oxidation.

The aim of this work was to perform the microencapsulation of rosmarinic acid (RA) in carboxymethyl cellulose (CMC) and ethyl cellulose (EC) polymers, using as encapsulation methods spray drying and o/w solvent evaporation, respectively. After encapsulation the microparticles were characterized in terms of shape and size distribution, using scanning electron microscopy and coulter counter-LS 230 particle size analyzer, respectively. Controlled release studies were performed in water and octanol, during 24 hours to simulate cosmetic vehicles. The antioxidant capacity of the encapsulated rosmarinic acid was evaluated using ABTS radical scavenging assay.

UV-Vis spectrophotometry analytical method was used to quantify the amount of rosmarinic acid released from microparticles. All the measurements were performed at 324 nm. The limit of quantification (LOQ) in water and octanol was 1.66 and 0.700 mg/L of rosmarinic acid, respectively while, the limit of detection (LOD) in water and octanol was 0.497 and 0.210 mg/L of rosmarinic acid, respectively. The analytical method was validated for both mediums with correlation coefficients greater than 0.995. The intermediate precision and repeatability only evidenced coefficients of variation (%CV) greater than 5% for the lowest RA concentration (3 mg/L). Accuracy studies showed recovery values (%R) greater than 97% for all the concentrations except for 9 mg/L of RA in water (94.10%). Therefore, no significant variations in the measurements were observed which demonstrates that the analytical method reproduced reliable results.

Regarding particles size, considering the number distribution, CMC and CMC-RA particles had a mean size of 0.156 and 0.358 µm, respectively while, taking into account the volume distribution, CMC and CMC-RA microparticles had mean size of 13.49 and 10.90 µm, respectively. These results suggest a size heterogeneity of the powder or agglomeration. CMC-RA microparticles prepared by spray drying presented a smooth surface and a spherical regular shape. EC-RA microparticles prepared by o/w solvent evaporation presented an irregular shape as well as some roughness on the surface.

O/W solvent evaporation method presented high product yield (95.4%) but low encapsulation efficiency (6.87%). On the other hand, spray drying microparticles showed higher encapsulation efficiency (90.8%) but lower product yield (38.4%).

The controlled release studies in water revealed that CMC-RA microparticles had a faster release (17.5 mg RA/g microparticles at 2 h) than EC-RA microparticles (2.41 mg RA/g microparticles at 2 h). In addition, EC-RA microparticles only released around 20% of their drug content in water. The release of EC-RA microparticles in octanol (58% at 2 h) was faster than in water (13%).

Finally, the antioxidant activity of encapsulated rosmarinic acid in CMC (208 μM TE) and EC (197 μM TE) was slightly higher than the respective free rosmarinic acid solution.

These preliminary studies of rosmarinic acid controlled release, using CMC and EC as wall materials, suggest that they can be used for its microencapsulation since they retarded the release of the antioxidant and they did not reduce its antioxidant activity.

Key words: rosmarinic acid, antioxidants, cosmetics, delivery systems, microencapsulation, spray drying, solvent evaporation, carboxymethyl cellulose, ethyl cellulose, controlled release

Resumo

Os antioxidantes são moléculas capazes de se oxidarem a elas mesmas prevenindo a oxidação de outras. Na cosmética, os antioxidantes poderão ser usados para retardar o envelhecimento na pele causado pelo stress oxidativo. Para além disso, este ingrediente poderá também ser utilizado como conservante combatendo a possível oxidação de compostos lipídicos presentes no produto ou outros ingredientes sensíveis à oxidação.

O trabalho realizado consistiu na microencapsulação de ácido rosmarínico (RA) em polímeros de carboximetil celulose (CMC) e etil celulose (EC), utilizando as técnicas de secagem por atomização e evaporação do solvente de emulsões óleo em água, respetivamente. Após a microencapsulação, as micropartículas formadas foram caracterizadas relativamente à sua distribuição em tamanho e forma, usando um analisador de tamanho de partículas coulter counter-LS 230 e microscopia eletrónica de varrimento (SEM), respetivamente. Os estudos de libertação controlada do RA encapsulado foram realizados em água e octanol, durante 24 horas de forma a simular as condições de formulações cosméticas simples. A capacidade antioxidante do ácido rosmarínico foi também avaliada recorrendo ao ensaio de eliminação de radicais de ABTS.

A espectrofotometria ultravioleta-visível foi utilizada como método analítico para quantificar o RA libertado das micropartículas. Todas as medições foram efetuadas com o comprimento de onda de 324 nm. O método analítico foi validado para ambos os meios de libertação com coeficientes de correlação superiores a 0,995. Os limites de quantificação (LOQ) em água e octanol obtidos foram 1,66 e 0,700 mg/L de RA, respetivamente. Os limites de deteção em água (LOD) e octanol foram 0,497 e 0,210 mg/L de RA, respetivamente. A precisão intermédia e repetibilidade apenas apresentaram valores de coeficientes de variação (CV%) superiores a 5% para a concentração mais baixa (3 mg/L). A exatidão apresentou valores de recuperação (R%) acima dos 97% exceto para a concentração de 9 mg/L de RA em água (94,10%). Assim, não foram observadas variações significativas nas medições o que demonstra a fiabilidade dos resultados obtidos usando este método.

Relativamente à distribuição de tamanhos, considerando uma distribuição em número, as partículas de CMC sem antioxidante e de CMC com RA revelaram um diâmetro médio de 0,156 e 0,358 µm, respetivamente. Considerando uma distribuição em volume, as micropartículas vazias de CMC e contendo RA encapsulado apresentaram um diâmetro médio 13,49 e 10,90 µm, respetivamente. Estes resultados sugerem uma heterogeneidade do tamanho das partículas do pó. Pela analise de SEM verificou-se que as micropartículas de CMC com RA apresentaram uma geometria esférica e uma superfície lisa. Por outro lado, as micropartículas de EC com RA demonstraram uma forma irregular e uma superfície mais rugosa.

A técnica de evaporação do solvente de emulsões óleo em água apresentou elevados rendimentos (95,4%), mas eficiências de encapsulação baixas (6,87%). Por outro lado, a secagem por atomização demonstrou altos valores de eficiência de encapsulação (90,8%), mas valores relativamente baixos de rendimento (38,4%).

Os ensaios de libertação controlada em água revelaram que as micropartículas de CMC com RA conduziram a uma libertação mais rápida do antioxidante (17,5 mg RA/g micropartículas em 2 h) que as micropartículas de EC (2,41 mg RA/g micropartículas em 2 h). Para além disso, as micropartículas de EC apenas libertaram aproximadamente 20% do seu conteúdo em RA no fim das 24 horas de ensaio. A libertação do antioxidante das micropartículas de EC em octanol (58% em 2 h) foi mais rápida que na água (13%).

Por último, a atividade antioxidante do RA encapsulado em CMC (208 µM TE) e em EC (197 µM TE) foi ligeiramente superior a uma solução contendo RA que não sofreu o processo de encapsulação. Assim, estes estudos preliminares de libertação controlada de RA, utilizando CMC e EC como agentes encapsulantes, sugerem que a microencapsulação poderá ser uma boa solução para retardar a libertação do antioxidante sem comprometer o ser desempenho.

Palavras chave: ácido rosmarínico, antioxidantes, cosmética, microencapsulação, secagem por atomização, evaporação do solvente, carboximetil celulose, etil celulose, libertação controlada

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Glossary

% CV	Coefficient of variation
%R	Recovery percentage
~y	Absorbance average
4CL	Hydroxycinnamate coenzyme A
A	Regression slope
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
B	Intercept of the regression
C4H	Cinnamic acid 4 hydroxylase
CAGR	Compound annual growth rate
CMC	Carboxymethyl cellulose
CMC-RA	Carboxymethyl cellulose rosmarinic acid loaded microparticles
DL	Drug loading
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC	Ethyl cellulose
EC-RA	Ethyl cellulose rosmarinic acid loaded microparticles
EE	Encapsulation efficiency
HPLC	High-performance liquid chromatography
HPPR	4 hydroxyphenylpyruvate reductase
K_{ow}	Coefficient of partition octanol- water
LMWA	Low molecular weight antioxidants
LOD	Limit of Detection
LOQ	Limit of quantification
NLC	Nanostructured lipid carriers
PAL	Phenylalanine ammonia- lyase
PBS	Phosphate-buffered saline
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly(l-lactic acid)
PVA	Polyvinyl alcohol
R	Coefficient of correlation
RA	Rosmarinic acid
ROS	Reactive oxygen species
S_a	Slop standard deviation
S_b	Intercept standard deviation

SC	Stratum corneum
SEM	Scanning electron microscopy
SLN	Solid lipid nanoparticles
S_y	Absorbance standard deviation
T	Confidence interval of 95%
TAT	Tyrosine aminotransferase
TE	Trolox Equivalent

Objectives and thesis organization

The aim of the experimental work was to perform the microencapsulation of rosmarinic acid (RA) in carboxymethyl cellulose (CMC) and ethyl cellulose (EC) polymers, using as encapsulation methods spray drying and o/w solvent evaporation, respectively. After encapsulation the microparticles were characterized in terms of shape and size distribution, using scanning electron microscopy and coulter counter-LS 230 particle size analyzer, respectively. Controlled release studies were performed in water and octanol, during 24 hours to simulate cosmetic vehicles. The antioxidant capacity of the encapsulated rosmarinic acid was evaluated using ABTS radical scavenging assay.

This master thesis is organized in six chapters: introduction, state of the art, equipment and reagents, methods, results and discussion, conclusion. Other sections such as background motivation and future work and limitations are also presented. Chapter I, introduction, presents the basic explanation of the importance of antioxidants focusing in rosmarinic acid. In addition, it also describes some of the concepts of the transdermal barrier and the basic fundamentals of microencapsulation. Some of the challenges behind the development of a delivery system are also mention during the whole chapter. Chapter II, state of the art, compiles and describes some of the information presented in appendix 2 providing the reader with the description of some research articles using delivery systems for cosmetic application. Chapter III, equipment and reagents, presents the equipment and reagents used in the experimental work. Chapter IV, methods, describes the methods used to perform the work as well as the conditions that allowed the validation of the analytical method. Chapter V, results and discussion, presents the main results regarding the analytical method validation, characterization of the microparticles, controlled release studies and antioxidant activity assessments. Finally, chapter VI, conclusion, presents the main conclusions of this work. All the references used in this thesis may be consulted in references section as well as some additional information in appendix 1, 2, 3 and 4.

Background motivation

Cosmetics and personal care products are market sectors that represent billions of dollars worldwide. United States of America is one of the main countries in this industry with a revenue stream around 60.58 billion U.S dollars and employing about 62,450 people, by 2015 [1]. On the other side, in Europe, Germany was the greatest country in 2014, with a sales volume of approximately 13 billion euros, followed by France (10.6 billion euros) and United Kingdom (10.4 billion euros) [2]. Among the different cosmetic categories, skincare products are clearly dominant with a market share of 35.3% [3].

Despite the expected expansion of cosmetics industry, with an annual growth rate of 3.6% in 2014, a constant innovation is crucial to catch the attention of new consumers and keep the current consumers loyal to specific brands [1]. In order to do so, new ingredients with different and promising functions have been studied, in combination with recent technologies and formulations to extend the product shelf-life and improve its performance.

Antioxidants are extensively used and advertised on cosmetic products that claim, most of the times, anti-ageing properties (Appendix 1). This ingredient can be either synthetic or natural, easily found in a different number of plants, fruits, grains [4]. Although synthetic antioxidants represent around 60% of the total consumption of antioxidants in several industries, the demand for natural compounds is increasing [5]. Besides its health benefits, antioxidants may be used as product preservatives as well. However, these molecules are unstable and may be sensitive to light, pH, temperature, oxygen which can lead to degradation and loss of effectiveness of the product. For topical application, they may also have some difficulties crossing the transdermal barrier due to their physical chemical properties and the heterogeneous skin constitution [6].

Delivery systems are innovative technologies used to ensure the stabilization, protection and targeted release of an active ingredient. Some of the most common delivery systems used in cosmetics and personal care products comprise liposomes, microparticles, emulsions and it is expected that their global market will reach 543,373.2 thousand US dollars in 2020 [7]. Microencapsulation is mostly used to protect and stabilize the active agent by surrounding it with shell material. Several methods and materials may be used to entrap the active ingredient leading to the formation of different types of particles. The materials and operational conditions must be chosen and optimized according to the final application of the microparticle.

There are several companies that produce microparticles to incorporate in cosmetic formulations [7]. Besides that, a great number of published articles and patents were also found, suggesting a crescent and common interest of this technology by scientific community and industries [8]–[10].

I. Introduction

I.1. Antioxidants

Antioxidants are molecules capable of oxidizing themselves instead or before other molecules. They are compounds or systems that can interact with free radicals and terminate a chain reaction before vital molecules are damaged [4]. The use of antioxidants is reported in food, cosmetics, beverages, pharmaceuticals and even in animal feed industry. They may be used as supplements and active ingredients, with health benefits, or as preservatives [5].

Reactive oxygen species (ROS) are one of the major causes of oxidative stress which enhances the skin ageing process [11]. Intrinsic ageing is associated with natural process of ageing while extrinsic ageing is related to external factors (e.g. air pollution, UV radiation, pathogenic microorganisms) that affect the ageing process. Photo ageing is probably the main reason of ROS production and acceleration of skin ageing process. Several potential targets can be found to interact with ROS in the skin (e.g. lipids, DNA, proteins) [12]. Figure 1 presents some of the potential oxidative stress effects in the skin.

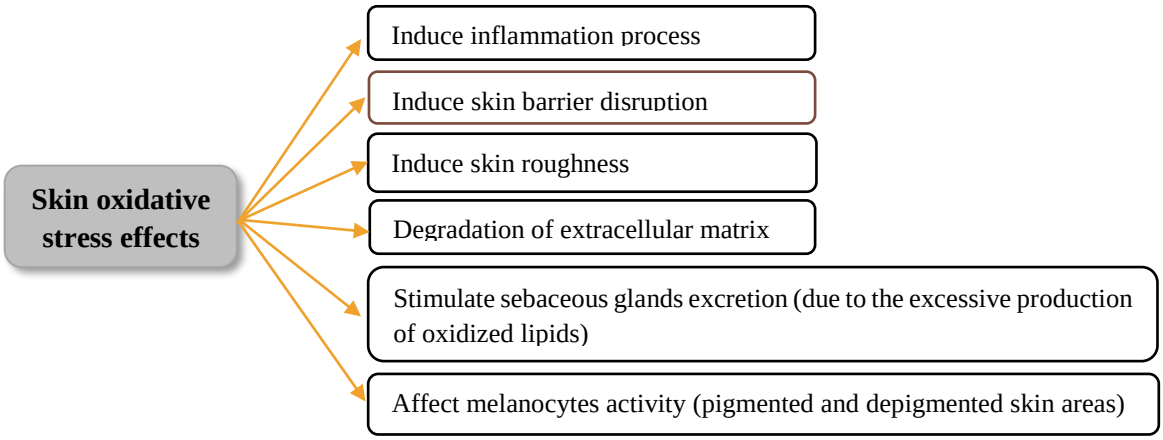


Figure 1 - Possible skin oxidative stress effects [11].

Antioxidant molecules can be enzymes or low molecular weight antioxidants (LMWA) that can act by donating an electron to reactive species interrupting the radical chain reaction, by preventing the reactive oxidants formation, by acting as metal chelators, oxidative enzyme inhibitors or antioxidant enzyme cofactor's [13], [14]. LMVA include a great number of substances and extracts that can be obtained from a variety of plants, grains, fruits and used in cosmetic industry. Table 1 presents some examples of natural antioxidants as well as their natural sources. Rosmarinic acid was the antioxidant studied in this work.

Antioxidants can also be used as preservatives avoiding the rancidity of lipid ingredients. In fact, lipid oxidation (Figure 2) is present in chemical products but also in the human body. In this way, antioxidants may have multi functions when present in a product. In the initiation phase of lipid oxidation, the number of radicals is expanded. During the propagation phase, molecular oxygen and fatty acid radicals react leading to the

formation of hydroperoxide products. Hydroperoxide are unstable and can degrade to produce radicals that will accelerate the propagation reaction. The termination phase is dominated by reactions between radicals [15]. Antioxidants can prevent lipid oxidation by reacting with lipid and peroxy radicals, converting them to more stable and non-radical products. In addition, antioxidants are also able to reduce hydroperoxides to hydroxy compounds, deplete molecular oxygen, deactivate singlet oxygen, remove prooxidative metal ions, replenish hydrogen to other antioxidants and absorb UV light [14], [16].

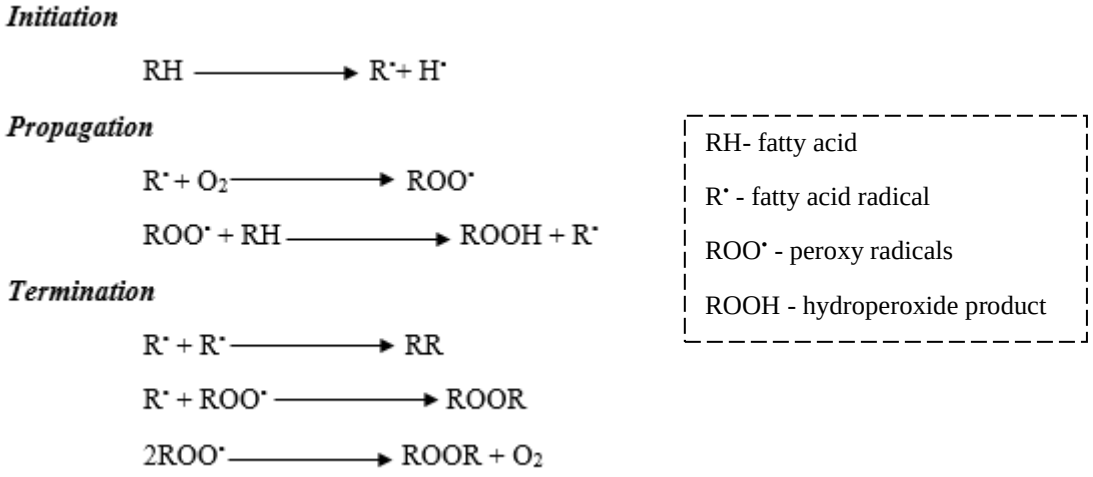
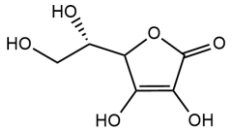
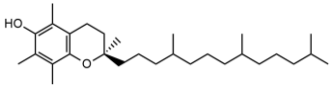
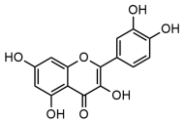
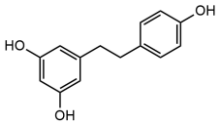
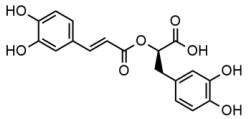
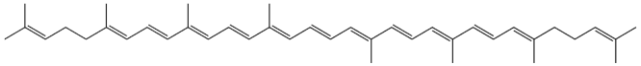
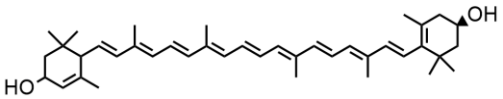


Figure 2 – Example of autoxidation mechanism [16]

Some studies report that neurodegenerative diseases like Alzheimer or Parkinson can be caused by oxidation of polyunsaturated lipids of cell membranes by free radicals. In this way, antioxidants have been studied as possible therapy for this kind of diseases [17], [18]. According to literature, antioxidants can also be involved in cancer treatments since the production of reactive oxygen species is altered during tumorigenesis [19]. Other mechanisms and implications in cancer disease are also reported in literature [18], [20], [21]. In addition, antioxidants play a role in diabetes disease, cardiovascular and hepatic diseases. Besides, they have anti-inflammatory, immuno-stimulant and anti-microbial properties as well [18], [22–26].

Nevertheless, antioxidants have some limitations that narrow their inclusion on all type of products mentioned above. According to literature, the stability of antioxidants is influenced by light, pH, temperature and oxygen [27–29]. Additionally, they can react with other matrix compounds, degrade and lose their activity [30]. Some antioxidants are used in the form of extracts which can give some taste or smell to the product as well [31], [32]. One of the main challenges for antioxidants, mainly for topical application, remains in the ability of crossing the dermal barrier. This difficulty is explained by the distinct characteristics that are associated to the different skin layers [33], [34].

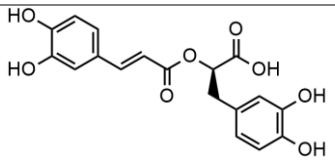
Table 1 - Examples of the mostly used natural antioxidants in cosmetic formulation

Classification	Antioxidant	Source	Chemical Structure	K_{ow}	Reference
Vitamins	Vitamin C	Apple, bayberry, broccoli, citrus peel, garlic, peppermint, spearmint		1.850	[4], [35–37]
	Vitamin E	Olives and olive oil, palm oil, pumpkin seeds, sunflower seeds and sunflower oil		12.180	[4], [36], [38]
Polyphenols	Quercetin	Black pepper, onions, curly kale, leeks, broccoli, blueberry, red wine and tea		1.5	[6], [39–42]
	Resveratrol	Red wine, grape berry skins and seeds, peanuts, dried roots of plant *Polygonum cuspidatum*		3.10	[43], [44]
	Rosmarinic acid	Oregano, rosemary, marjoram, clary sage, thyme, basil		1.82	[45]
Carotenoids	Lycopene	Apricots, grapefruit, guava, watermelon, papaya and carrots		16.6	[4], [46–48]
	Lutein	Spinach, leaf lettuce, peas, oranges, kale, zea mays, carrot		7.9	[4], [49–52]

I.1.1. Rosmarinic Acid

Rosmarinic acid (RA) is a polyphenol found in a wide variety of plants from the Lamiaceae family: oregano (*Origanum vulgare* L.) rosemary (*Rosmarinus officinalis* L.) , marjoram (*Origanum majorana* L.), clary sage (*Salvia sclarea* L.) , thyme (*Thymus vulgaris* L.), basil (*Ocimum basilicum* L.) [45]. This compound was first isolated in 1958 by two Italian chemistries, Scarpati and Oriente [53]. Table 2 presents some chemical and physical properties of rosmarinic acid.

Table 2 - Physical and Chemical properties of rosmarinic acid [45]

Chemical structure	
IUPAC name	(2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid
Molecular Formula	$C_{18}H_{16}O_8$
Melting point (ºC)	171-175
Vapor pressure at 25 ºC (mmHg)	$1.1x10^{-13}$
log K_{ow}	1.82
Solubility (mg/mL)	DMSO: 25 DMF: 25 Ethanol: 25 PBS:15 Water:1.0

Rosmarinic Acid is an ester of caffeic acid and d 3,4-dihydroxyphenyllactic acid. The biosynthesis of this antioxidant is initiated by two parallel pathways - phenylpropanoid pathway and tyrosine derived pathway. The first via comprises three steps, using phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and hydroxycinnamate coenzyme A ligase (4CL). The second via involves two successive steps using tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate reductase (HPPR). Subsequently, the two resulting products (4-coumaroyl-CoA and 4-hydroxyphenyllactic acid) act as substrates for rosmarinic acid precursor (4-coumaroyl-4'-hydroxyphenyllactic acid) and then to the final conversion to rosmarinic acid [54]. The chemical synthesis of this antioxidant was first achieved in 1991 [53].

According to literature, there are several benefits related to rosmarinic acid besides its strong antioxidant activity: anti-inflammatory, anti-mutagenic, anti-bacterial, anti-viral, anti-cholinesterase and anti-tumor effects [53], [55]. Its antioxidant activity is higher than vitamin E and it also acts as scavenger of free radical [56]. During oxidation reaction, rosmarinic acid molecules may be transformed to quinones (Figure 3) [57].

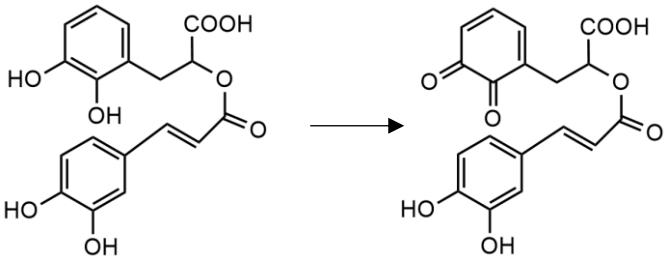


Figure 3 - Example of rosmarinic acid oxidation to the respective quinone

Studies show that rosmarinic acid can be involved in therapies related to ocular disease, since data suggests rosmarinic acid could be a potent inhibitor of retinal neovascularization and may be used in the vasoproliferative retinopathies treatments [30]. Furthermore, rosmarinic acid have proven neuroprotective action in animal models of neurodegenerative diseases such as Alzheimer and Parkinson. Literature also shows the effectiveness of this antioxidant against memory deficits induced by permanent focal cerebral ischemia in mice [58]. Rosmarinic acid can also play a role in epilepsy disease, liver/brain damage following ischemia and reperfusion, rheumatoid and arthritis disease [59–61]. Several studies report its involvement in cancer treatments such as colon cancer (tested in mice) or leukemia (tested *in vitro*) [62], [63]. The use of rosmarinic acid as preservative can also be beneficial for cosmetic or food products. *Thymus nummularius* methanol extract containing rosmarinic acid revealed antimicrobial activity against pathogenic microorganisms such *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *C. albicans* [64]. For dermatologic application, published articles evidenced the role of rosmarinic acid in melanin production, which can be used as a therapeutic agent for skin pigmentation disorders, and it can have a role in protection against photo carcinogenesis [65–67]. Atopic dermatitis treatment was also evaluated in mice using rosmarinic acid and it presented positive results [60]. The photoprotective effect of rosmarinic acid in UVA induced changes in human keratinocytes was also verified. Rosmarinic acid decreased the production of ROS, DNA damage and increased the keratinocytes cell viability [68]. Therefore, this antioxidant is a substance of interest for cosmetic industry.

However, it has some particularities that limit its use in cosmetics formulations (emulsions and creams): low water solubility, discoloration and instability [56]. The low K_{ow} (1.82) makes it difficult to cross the transdermal barrier in order to reach the deeper layers of the skin and once there, reduce the amount of ROS [34].

The use of delivery systems can overcome these limitations. Delivery systems may not only guarantee the protection and stabilization of rosmarinic acid during storage and manufacturing process, but also they may improve its physical chemical properties to facilitate the transport across biological barriers. The choice of the carrier material is crucial since it has to be biocompatible and it has to be able to solve all the limitation mentioned above.

I.2. Delivery systems

Nowadays, consumers are not only focused in cosmetic products that only promote their beauty. They are seeking for products claiming health benefits, the use of natural ingredients combined with recent technologies that can improve the cosmetic performance. Drug delivery systems are engineered technologies used to carry an active ingredient promoting a controlled and targeted delivery. Human skin acts as a barrier against the permeation of exogenous molecules. Drug delivery systems are able to enhance the permeation of the active ingredient through the skin layers, controlling its concentration in the formulation and on the skin. For cosmetics purpose, it is a major concern to keep the active ingredient in the superficial skin layers and avoid the systemic absorption [69]. Burst and sustained release are two major features that may be associated to cosmetic delivery systems. Burst release is important to improve the penetration of active molecule, while sustained release becomes important when the active ingredient is irritating at high concentrations, or to supply the skin for long periods of time [70]. The protection of the active ingredient is another advantage of using delivery systems. The shelf life demanded for a cosmetic product is usually no less than 2 years. Therefore, sensitive ingredients to external conditions like light, oxygen, heat must be protected to ensure the stability of the product. In addition, drug delivery systems can also prevent the reaction between the encapsulated ingredient and other molecules in the product matrix [71]. Several types of delivery systems can be found in cosmetic products (Table 3). They may be divided into vesicular systems (liposomes, niosomes, transfersomes, emulsions (microemulsions and nanoemulsions), particulate systems (micro particles, nanoparticles) [69].

Table 3 - Example of delivery systems used in cosmetics and their materials

Delivery system	Material	Ref.
Liposomes	***Lipids***: Soya phosphatidylcholine, dipalmityl phosphatidylcholine, distearyl phosphatidylcholine	[72]
Niosomes	***Nonionic surfactant:*** Polyoxyethylene alcohol, polyoxyethylene glycol alkyl ethers, alkyl ethoxylate, alkyl phenol ethoxylate, fatty acid alkanolamides, propylene oxide-modified polymethylsiloxane	[73]
Transfersomes	***Lipids:*** Soya phosphatidylcholine, egg phosphatidylcholine, dipalmityl phosphatidylcholine, distearyl phosphatidylcholine ***Edge activator***: Sodium cholate, sodium deoxy cholate, Tween 80, Span 80	[74]
Solid lipid Nanoparticles (SLN) & Nanostructured lipid carriers (NLC)	***Solid lipids:*** Tristearin, stearic acid, cetyl palmitate, cholesterol, Precirol® ATO 5, Compritol® 888 ATO, Dynasan®, 116, Dynasan® 118, Softisan® 154, Cutina® CP, Imwitor® 900 P, Geleol®, Gelot® 64, Emulcire® 61 ***Liquid lipids:*** Medium chain triglycerides, paraffin oil, 2-octyl dodecanol, oleic acid, squalene, isopropyl myristate, vitamin E, Miglyol® 812, Transcutol® HP, Labrafil Lipofile® WL 1349, Labrafac® PG, Lauroglycol® FCC, Capryol® 90	[75]

Table 3 - Example of delivery systems used in cosmetics and their materials

Delivery system	Material	Ref.
Polymeric Nanoparticles and microparticles	***Natural:*** Collagen, albumin, whey protein, casein, gelatin, chitosan, agarose, alginate, carrageenan, dextran, pectin, cyclodextrins, hyaluronic acid ***Synthetic:*** Poly(lactic acid), poly(glycolicacid), poly(lactic-co-glycolic acid), poly(ε-caprolactone), poly(alkylene succinates), poly(hydroxyl butyrate),	[76]

I.2.1. Nanoparticles and Microparticles

Polymeric microparticles were the main focus of this work. Nano/microparticles are used to entrap active ingredients (core material) inside or scattered in a surrounding material (shell material) [77]. The exact definition of nanoparticles and microparticles is not consensual among authors. Some researchers consider that nanoparticles size should range between 1 and 100 nm, while others claim that nanoparticles must range between 1-1000 nm [78], [79]. Nano/microparticle is a general term comprising both nano/microspheres and nano/microcapsules. Nano/microspheres consist of a homogenously dispersion of the active ingredient in the polymeric matrix whereas nano/microcapsules are reservoirs where distinct domains of core and wall material are present (Figure 4) [80]. One of the main purposes of this procedure is the creation of a physical barrier that avoids the contact of the active agent with the external matrix protecting sensitive substances from moisture, pH, light, oxygen and other molecules present in the matrix [81]. This barrier is also responsible for a controlled release of the active ingredient which can be regulated by heat, mechanical action, pH, biodegradation, diffusion and dissolution [82]. Nano/microparticles are often used to enhance the permeation of the active ingredient through the skin without the penetration of the particle. Nanoparticles, in particular, can be applied as preservatives and antibacterial agents in cosmetics [83].

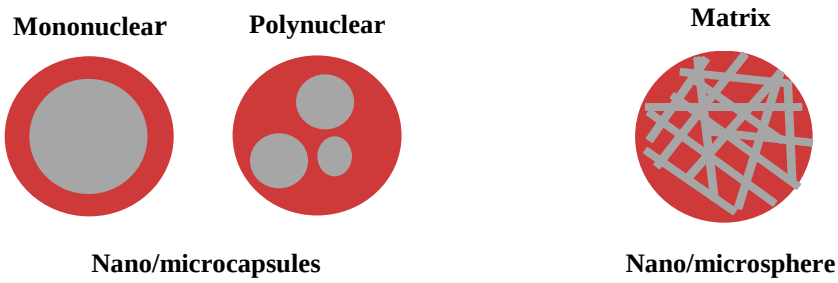


Figure 4 - Schematic representation of nano/microcapsules and nano/microsphere

The preparation of nanoparticles and microparticles may be performed by the same methods (i.e. evaporation or extraction of the solvent, interfacial polymerization, spray drying) with controlled operational conditions in order to obtain the desired product [84].

A wide variety of encapsulating agents can be used, extending from synthetic to natural. Its choice should be made according to the particle application, the selected core material, the physical and chemical stability, the required particle size, the release mechanism and manufacturing costs [82], [83]. Occasionally, a combination of wall materials is used to achieve the desired properties [84].

Nanoparticles have a higher tendency to aggregate due to their high surface area and the type of interaction that they can establish with each other [85]. Additionally, particles size also interferes with the release of the active ingredient. Active ingredient entrapped in smaller particles have greater access to the external phase which can lead to a higher release by diffusion, a faster penetration of water into the particle and a lower drug loading. The adsorption of molecules on the surface also occurs during particle formation and it is more accentuated the smaller the particle is. On the other hand, smaller particles may have a better binding for a unit of particle mass than larger ones which could be useful to adhere on the skin [86]. Transdermal barrier and the interactions of the different delivery systems with it is another critical aspect that must be considered.

I.3. Transdermal barrier

Human skin can be divided in three distinct layers: epidermis, dermis and subcutaneous layer (Figure 5). Epidermis is the outer layer (thickness: 50-150 µm) that has a protective role since it is in contact with the environment. It comprises several layers from the stratum corneum (upper layer) to the basal cell layer that are constantly being regenerated. The second layer of human skin is dermis and it composed of glycosaminoglycans, blood vessels, nerves, sweat glands, hair follicles and sebaceous glands. Elastin and collagen are here synthetized and they are responsible for the tensile strength, resilience and elasticity of the skin, respectively. The inner layer is hypodermis or subcutaneous layer which is composed of adipose tissue and it is responsible for the thermal control [34], [87], [88].

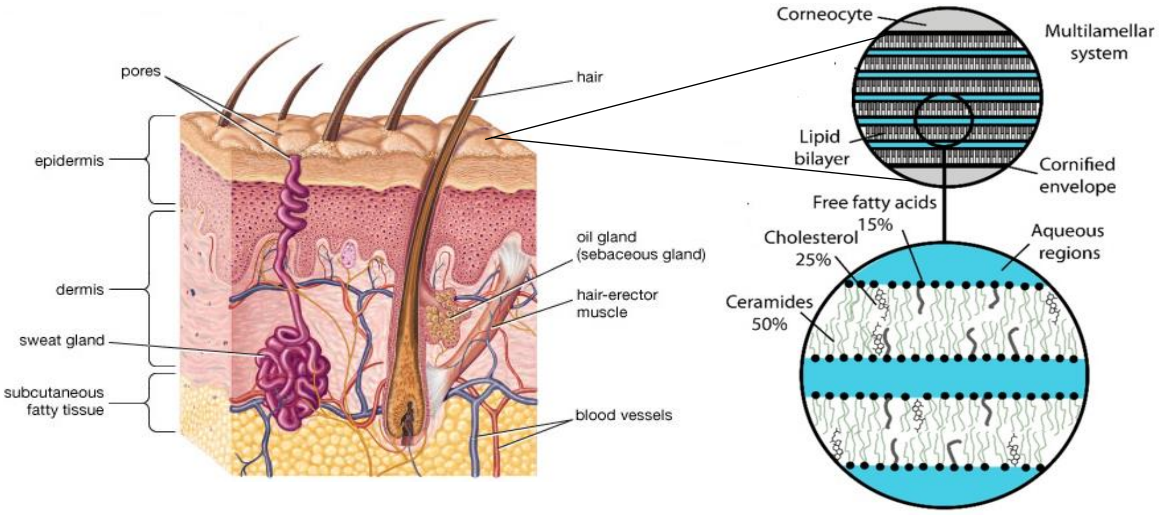


Figure 5 - Schematic representation of skin structure [192], [193].

Stratum corneum is composed of enucleated and completely differentiated keratinocytes cells, called corneocytes, mostly filled with keratin. Each corneocyte cell is surrounded by a lipid matrix and contains intact or degraded corneodesmosomes that are responsible for keeping cells together. According to literature, stratum corneum is composed of 15 layers of corneocytes with a diameter of 40 μm and thickness of 0.3 to 0.8 μm. Under air-dried conditions the gap between cells is 75 nm. Looking from the top to the inside, it is possible to observe that corneocytes have a hexagonal shape and are grouped in clusters up to 12 units [89]. Pathways between the clusters offer less resistance delimiting the intercorneocyte penetration pathway. Transcellular penetration pathways are not significant for transdermal passage [90]. Stratum corneum hydration is an important factor to consider in drug delivery and it may be regulated by the proteolysis of the corneocyte content. The degradation products consist in a mixtures of osmotically active amino acids that capture water and act as moisturizing factors.

The lipid matrix is composed of different types of lipids, i.e. cholesterol and its derivatives, ceramides, free fatty acids and triglycerides, that are responsible for its space organization. However, the basis of the lipid matrix structure consists in lamellar repeating hydrophilic and lipophilic bilayers where approximately 80% of the lipid are non-polar, with hydroxyl groups, able to settle hydrogen bond interactions with adjacent lipids or water [89]. As we go deeper in the skin layers the content in water increases: the lipophilic stratum corneum contains around 13% of water, the viable epidermis about 50% of water and the dermis around 70% of water [34]. In stratum corneum, hydrophilic regions are created mainly in the lateral cell junctions due to the non-planarity of the corneocytes outer membrane. Less ordered lipids and flexible hydrophobic chains are also found in interlamellar and linker regions. Such characteristics are extremely important for transepidermal diffusion of lipophilic and amphiphilic molecules since they guarantee the necessary space for migration. Hydrophilic molecules diffuse preferably “laterally” through the water filled inter lamellar spaces or the free spaces between the lamella and the corneocyte outer membrane [90]. The chemical composition of stratum corneum is also responsible for a pH gradient ranging from 4.5 and 5.5, in mammalian stratum corneum surfaces, to neutral values in stratum granulosum interface.

Therefore, the passage through the stratum corneum is limited to low molecular weight molecules (<500 Da), preferably uncharged and with K_{ow} values between 1 and 3. Regarding the use of delivery systems, they have to be smaller than 5–7 nm to potentially diffuse throughout the fluid lipid bilayers or smaller than 36 nm to eventually go through the aqueous pores [34], [89].

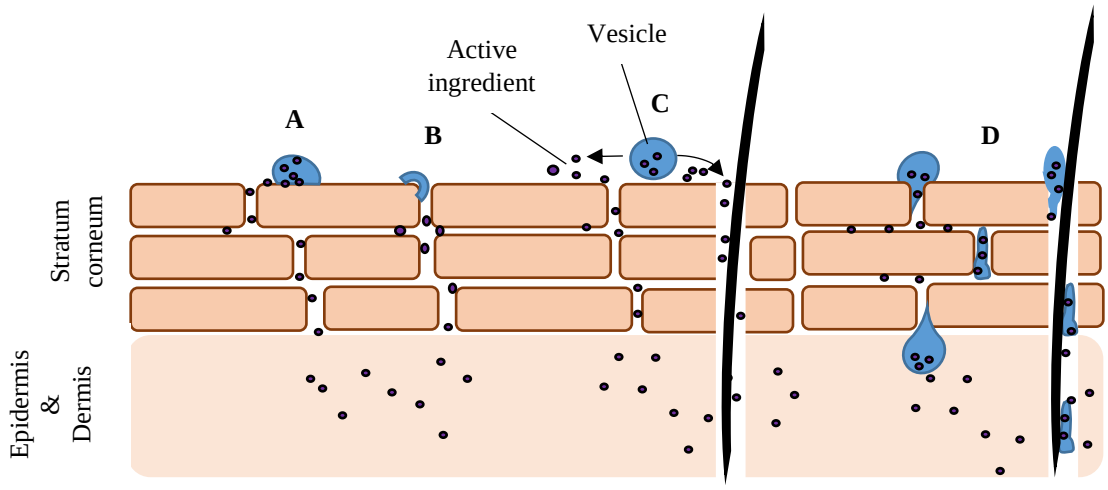
The penetration of active substances and nanoparticles is also possible through the pilosebaceous units and it has been demonstrating an increased interest. Pilosebaceous units are composed of the hair follicle, the adjoining arrector pili muscle and the associated sebaceous glands [91]. Their function is to synthetize and excrete a mixture of squalene, waxes, cholesterol derivatives, triglycerides fatty acids and cell debris, called sebum [34]. Sebum excretion acts as barrier that prevents the passage of molecules and particles to the inner layers. However, literature describes this non polar lipid mixture as a possible responsible for the uptake of some lipophilic molecules. Therefore, penetration agents may be able to disperse themselves in sebum [92].

Corneocytes barrier in the lower hair follicle infundibulum seems to be crumbly and smaller which makes this area more susceptible to penetration. Hair follicle infundibulum can also act as reservoir for nano and micro sized particles. It has been suggested that larger particles release high concentrations of the active ingredients to the follicle, while smaller particles (less than 40 nm) can pass through the disrupted skin barrier [93]. The hair follicle can be considered as an invagination of the epidermis extended deep to the dermis layer thus providing a greater improvement of penetration surface area. Cosmetic relevant drug targeting, through pilosebaceous units, can be directed to the sebaceous gland, viable skin epidermis, follicular papilla and hair matrix cells. The sebaceous gland area is associated to skin problems such as acne and it was recently found the existence of melanocytes stem cells. Follicular papilla and hair matrix are believed to be involved in hair growth and pigmentation. Regarding hair grow cycle, it can influence the penetration of active ingredients [92].

In order to improve skin delivery, penetration enhancers may be used. These substances may be used to increase the diffusion coefficient of the penetration molecule through the stratum corneum, to increase the effective concentration of the drug in the vehicle, to improve the partitioning between the formulation and stratum corneum. Examples of classic penetration enhancers consists in water, ethanol, dimethyl sulphoxide, laurocapram, oleic acid, surfactants. Ethanol is a commonly used solvent in cosmetic formulations. The mechanisms by which ethanol acts as penetration enhancer are different: as a solvent it can improve the solubility of an active ingredient in the vehicle; it can permeate and change the solubility properties of the stratum corneum; its rapid permeation or evaporation may alter the thermodynamic properties of the drug within the formulation; when used in high concentrations, it can extract some lipid fraction from the stratum corneum [94]. Recent studies suggest the use of peptides as penetration enhancers [95]. They may be considered as safer than traditional penetration enhancers since the last ones can cause skin irritation, cytotoxicity or alter irreversibly the skin barrier.

Vesicular delivery systems are frequently reported in cosmetic formulations. Their dimensions can range between the nano and the micro scale. Roughly speaking, they can be divided in deformable systems and rigid ones. Lipid vesicular systems can deliver the active ingredient just for releasing it from the vesicle, can enhance the transdermal passage due to interactions with stratum corneum, can fuse and exchange lipid material with stratum corneum, transferring the active ingredient to the skin (Figure 6) [96]. Intact vesicular skin penetration is associated to deformable systems since they can squeeze through the interclusters and intercorneocytes pathways using hydrating gradient as driving force [97], [98]. Deformable vesicles are often smaller than rigid ones which also justifies why they are frequently found deeper in the skin. The vesicular chemical composition is the key factor to establish the predominant mechanism by which a molecule is delivered through the skin.

Regarding rigid particulate systems (SLN, NLC, micro/nano particles), its penetration mostly depends on the particle size being its composition and charge less significant. SLN and NLC are claimed to form an occlusive film on skin surface that enhances the active ingredient penetration [89].



A- Fusion and lipid material exchange with stratum corneum
B- Enhancement of the transdermal passage
C- Free releasing from the vesicle
D- Intact vesicular skin penetration (only flexible vesicles)

Figure 6 - Possible mechanisms of action of vesicles as skin drug delivery systems [96]

I.4. Incorporation of delivery systems in cosmetic formulations

Generally cosmetic cream formulations are manufactured by preparing separately two distinct phases with the respective soluble ingredients. The homogenization step must be equipped with a vacuum line or a de-airing step should be added [99]. Thermal or volatile excipients must be added after the mixture is cooled down to a suitable temperature. The adjustment of pH can be performed in the end of the formulation or by adding the neutralizing agent generally to the aqueous phase. Some excipients can be sensitive to pH modifications therefore, a careful choice of the cosmetics ingredients should be made to ensure stability [100], [101]. Figure 7 represents a schematic example of the preparation of cream formulation.

A deliver system can be formulated in a liquid state or as powder. Regardless of the physical state, it has to be incorporated in a vehicle (i.e. cream, lotion) to be applied on the skin. After incorporation, it is necessary to guarantee a uniform product, sensorially attractive for the consumer and with a long term stability. Therefore, experimental formulations must be tested regarding its spreadability, rheological properties, color changes, pH changes, storage temperatures. Taking into account rheological properties, for instance, cosmetic formulations may be characterized by a non-Newtonian behavior since it is desired to decrease the viscosity of semi solid formulation when it is spread on the skin (i.e. an external force is applied). Furthermore, Newtonian systems such as liquids or emulsions may not form occlusion films, since they spread rapidly, affecting the effectiveness of the formulation [102]. A good stability after the rheological tests is also important since it indicates that the product will remain stable even while it is rubbed on skin. Additionally, *in vivo* tests should also be taken in order to evaluate the efficacy of the product or adverse irritation reaction that it may cause on the skin [103].

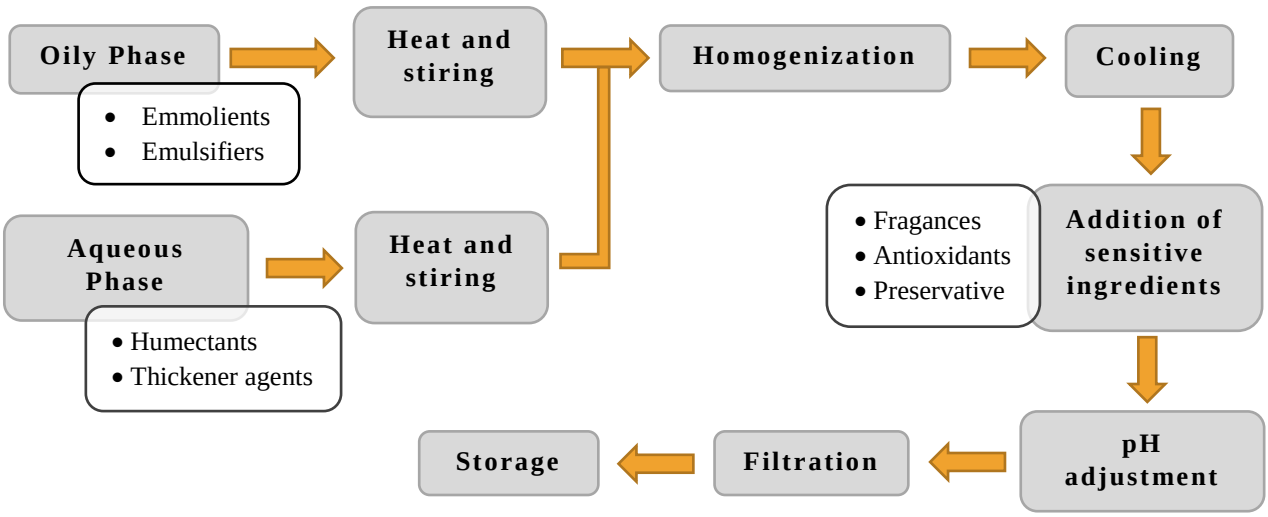


Figure 7 - Schematic representation of an example of preparation of a cosmetic cream formulation [99], [194]

The characterization of the delivery system can help to predict the behavior when it is incorporated in the cosmetic formulation. After prepared, delivery systems can be characterized concerning the size and morphology (scanning electron microscopy), mechanical properties (atomic force microscopy), thermal analysis (differential scanning calorimetry), zeta potential. Thermal analysis can be useful to determine the delivery system integrity after incorporation [104]. The size and zeta potential are two measurements that could help to prevent agglomeration since nano-sized particles and zeta potential values close to zero tend to aggregate [85]. After entrapment of an active ingredient into a delivery system (e.g. antioxidant), it is also advisable to evaluate its antioxidant activity since the process of encapsulation may affect its antioxidant properties. The protection of the antioxidant to light, oxygen or any other external factor should also be assessed for short or long periods of time.

The incorporation of a delivery system into a cosmetic formulation does not follow a single method or rule. Delivery systems can be incorporated in the final product, in the aqueous phase or in the oily phase. In addition, they may be incorporated in gels, dissolved in solutions or slurries that are further mixed. Considering SLN and NLC, the incorporation in the final product implies a reduction in the water content to incorporate an aqueous solution containing the lipid particles. Usually the cream formulations are produced, cooled and the particle lipid concentrate is added, stirring gently. This incorporation may lead to a viscosity increase and so, sometimes, it is necessary to reduce the lipid content of the initial formulation. Another method to produce SLN and NLC cosmetic formulations is to initially replace some water content by the lipid aqueous phase. To perform the last method, it is fundamental to assure the physical and chemical stability of the lipid particles during processing [105]. Another concern is the amount of active ingredient present in the final cosmetic formulation. Usually during the process, the formulations are diluted 10 to 20 times. In the final formulation, typically the particle concentration is between 2 - 4% while the active ingredient concentration should be 0.05 - 0.10% [106]. Therefore, the drug loading of the particles as well as the final proper concentrations of particles

and active ingredient must be considered and combined. Polymeric microparticles and nanoparticles may be added to any phase of the cosmetic formulation, according to its affinity to form a dispersion and avoid agglomeration. They can be added as a powder, a slurry or a wet cake. Powder microparticles can be directly incorporated or firstly dispersed in a solvent. Mechanical stirring and temperature are important factors to take into account since they can influence the integrity of the particle and lead to the release of the active encapsulated ingredient during manufacturing [107]. Microparticles will be further discussed to better understand some of the fundamentals of this type of delivery system since it is the main objective of this work.

I.5. Microencapsulation

Although several delivery systems may present micro scale dimensions, the concept of microencapsulation is usually intensively associated to the formation of polymeric microparticles. Microencapsulation techniques have been used worldwide and they have numerous applications in food, pharmaceutical, household products, cosmetics, agrochemicals etc. Some examples of the utilization of microparticles include carbonless paper, ‘‘scratch and sniff’’ fragrance sampling, ‘‘intelligent’’ textiles, controlled release of drugs and cosmetic active agents [82].

Microencapsulation has many advantages comparing to non-encapsulated substances since it allows the protection and stabilization of the core material and its controlled, timed and targeted release [108]. Product appearance and flow properties may also be improved, enhancing its handling, usage and storage. Undesirable organoleptic properties can be masked and the evaporation of volatile ingredients can be controlled using microencapsulation. This technique can also be used to reduce the amount of ingredients in formulation being a cost saving alternative [109].

Cosmetic industry represented 8% of microencapsulation market in 2013 [82]. Several companies are involved in the production of microparticles for a wide range of applications: Ronald T. Dodge Company, Lipo Technologies, Evonik Industries AG, GAT Microencapsulation GmbH. The market is expected to growth at a compound annual growth rate (CAGR) of 9.7% from 2014 to 2020 [110]. Microencapsulation is a suitable technique to solve some of the cosmetic ingredients (e.g. antioxidants) limitations since it protects sensitive substances prolonging the shelf life and stability of the product. It can also control the release of the active ingredient improving its penetration across the skin.

I.5.1. Microencapsulation methods

There are several methods for microencapsulation however its choice should be well considered and analyzed. The microencapsulation method should be selected based on the intended particle size, the biodegradability and biocompatibility of the particles, the physicochemical properties of the core and shell material, the final application of the particles, the proposed mechanism for core material release, the production scale and the processing cost. Several variables can also influence the formation of the microparticles.

Therefore, an optimization of the microencapsulation process should be performed in order to select the proper materials and conditions to get the desired outcome.

Generally, microencapsulation techniques can be divided into two major categories: chemical and physical methods; the latter one can be subdivided into physicochemical and physico-mechanical techniques. Chemical methods comprise interfacial, emulsion and suspension polymerization. Physicochemical methods are for instance, solvent evaporation /extraction, coacervation, sol-gel encapsulation, ionotropic gelation. Spray drying, spray chilling, fluid bed coating may be considered physical methods [111–113].

After microencapsulation, microparticles should be characterized in order to evaluate the encapsulation method. Some of the basic process aspects are the encapsulation efficiency, drug loading capacity and the product yield. Encapsulation efficiency (EE) refers to the amount of active ingredient associated with the particles comparing to the feed solution. Drug loading (DL) refers to the amount of the active agent present in the microparticles. Yield product is the ratio between the output mass obtained and the initial solid content of the feed solution [114]. Since the ratio between the core material and the encapsulating agent can be considered constant during spray drying, EE should be calculated indirectly. Free RA is determined by measure the amount of rosmarinic acid right after dispersion of the microparticles in the solvent. However, for solvent evaporation techniques the same consideration cannot be taken since some antioxidant can escape to the aqueous phase. Therefore, it is only possible to calculate EE directly. Table 4 presents a sum up of the last information.

Table 4 - Expression to calculate encapsulating efficiency, drug loading and product yield for spray drying and solvent evaporation methods

	Spray drying	**Solvent evaporation**
Encapsulation Efficiency (%)	$\frac{\text{Initial RA weight} - \text{Free RA weight} \times 100}{\text{Initial RA weight}}$	$\frac{\text{Entrapped RA weight} \times 100}{\text{Initial RA weight}}$
Drug loading (%)	$\frac{\text{RA weight in microparticles} \times 100}{\text{Microparticles weight}}$	
Product yield (%)	$\frac{\text{Output mass} \times 100}{\text{Input mass}}$	

I.5.1.1. Spray drying and solvent evaporation

Spray drying is probably the mostly used microencapsulation technique (Figure 8). A solution, a suspension or an emulsion containing the core material and the shell material is homogenized and then fed to a spray drying equipment. Then, the process can be divided in three steps: (1) atomization of the liquid solution (2) the contact of the fine droplets with a hot gas stream to evaporate the solvent (3) the separation and collection of the powder. Commercially available atomization systems consist of pressure nozzles, centrifugal atomizer, kinetic energy nozzle, and sonic energy atomizer. The spray drying operation mode can be in counter

current, co-current or a combination of both. The use of co-current operation mode is suitable for heat sensitive products since the solvent evaporation is fast with a very short residence time. The counter current mode is suitable for non-heat sensitive products and has a more efficient heat utilization. The combination of both operation mode is usually associated to smaller spray dryers. To achieve good encapsulation efficiency operational parameters like feed temperature, air inlet temperature and air outlet temperature should be optimized. The air inlet temperature is usually from 150 to 220 °C and air outlet temperature is from 50 to 80 °C. The drying times are in range of 5–100 s and in well design systems 15–30 s is a fair time [115]. Spray drying is a relatively simple process, with an operating low cost, easy to scale up and it can operate in continuous. However, some of the main drawback of this method are the high cost of the equipment, the low overall thermal efficiency and the possibility of loss of low-boiling point substances. The final product may not have a uniform size and may need further processing [82], [113].

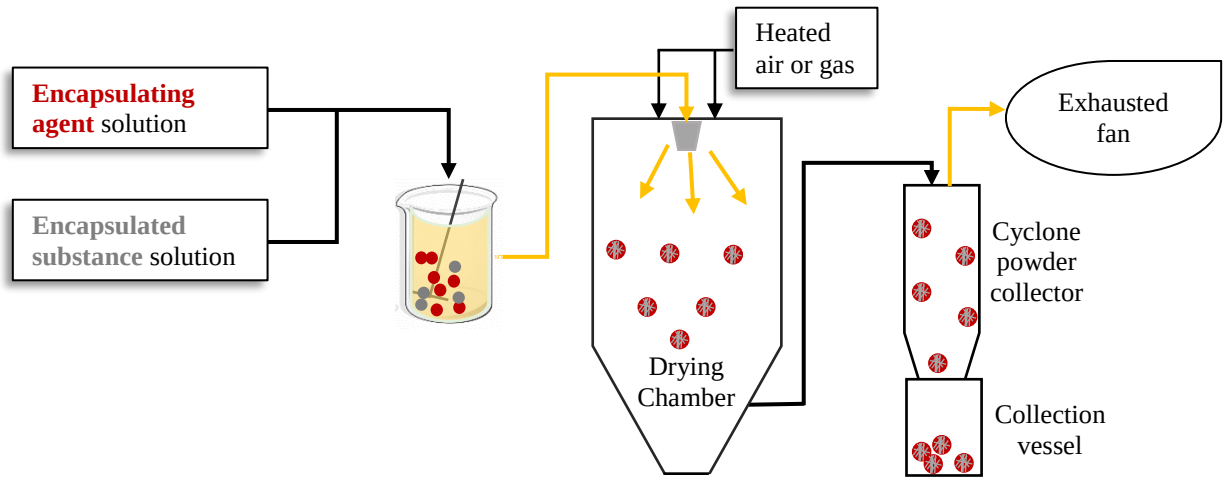


Figure 8 - Schematic representation of a spray dryer process

Solvent evaporation is a simple method frequently used since it allows the encapsulation of hydrophobic and hydrophilic substances. In this method, the polymer is dissolved in a water immiscible solvent and the encapsulated substance is dispersed or dissolved in the mixture (Figure 9). The resulting solution or dispersion is then slowly added to a water phase, frequently in a presence of an emulsifier agent, under stirring, to form an emulsion. To form the solid microparticle the solvent must now diffuse into the aqueous phase and evaporate. The microparticles can be further washed and collected by filtration, centrifugation and then, dried. Several factors affect the formation of microparticles which should be thus optimized. The surfactant ratio, the rate of solvent evaporation, the polymer molecular weight, the agitation rate, the organic phase volume are some examples of parameters than can interfere with the microparticle formation [116]. Considering the solvent evaporation method there are two specific techniques that are widely employed: single o/w emulsion and double w/o/w emulsion (Figure 9). In the first an oily phase containing the encapsulating agent and the core substance is added to the aqueous phase. In double emulsion, a first w/o emulsion is prepared and then it

is added to the aqueous phase to form a w/o/w emulsion. Double emulsion technique was developed to entrap hydrophilic molecules since they have tendency to escape to the aqueous phase when the single emulsion technique is used. Solvent evaporation method allows the incorporation of thermosensitive drugs since it does not require high temperatures. Some of the main drawback of this method is the use of high amounts of solvents, the low drug encapsulating efficiency and the hard scale up [112], [117].

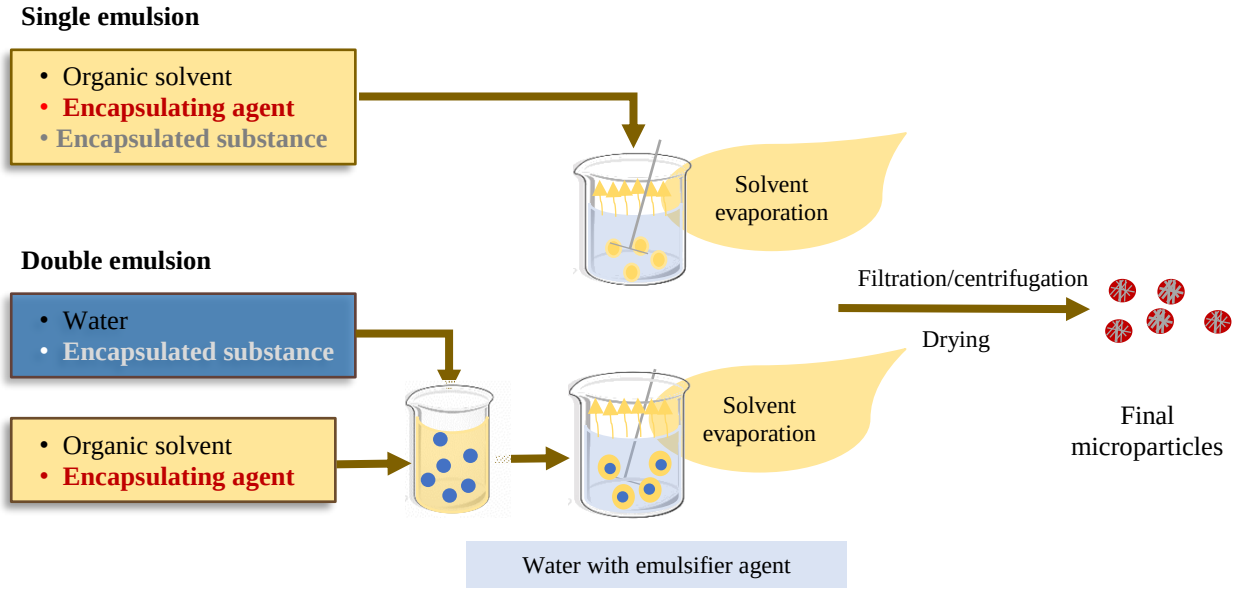


Figure 9 - Schematic representation of solvent evaporation method by single and double emulsion

I.5.2. Encapsulating agent

The encapsulating agent selection is a very important factor to consider during microencapsulation process since it affects its efficiency and the microparticles stability. However, most of the times the selection is made by trial-error procedures due to the unpredictable behavior of the formed complex [115].

The selection of the wall material should ponder the physicochemical properties of both core (e.g. porosity, solubility) and shell material (e.g. viscosity, mechanical properties). Furthermore, the wall material should not be soluble or react with the core. Besides the particle size, the final application of the microparticle is an important criterion to select the wall material. Different applications require different degrees of protection of the core and different release mechanisms of the active agent [118]. Sometimes one shell material does not have all the required characteristics so a combination of encapsulation agents may be used.

Wall materials can be classified as natural, semi-synthetics and synthetic polymers. Natural wall materials can be divided into carbohydrates (e.g. starch, maltodextrin, cyclodextrin), gums (e.g. arabic gum, acacia gum, alginate, carrageenan), proteins (e.g., gelatin, casein, soy protein). Synthetic shell material can be classified in polyesters (poly (lactic-co-glycolic acid), poly(ε-caprolactone), poly (alkylene succinates)), acrylic polymers (poly (methyl methacrylate), poly hydro(ethylmethacrylate), polymethacrylates),

phosphorous-based polymers and polyamides [76]. In a less extent, silicates and clays can also be used as encapsulating agents [82].

In order to use in cosmetic applications, wall materials should be biocompatible and once in contact with human body, they must not induce any adverse reaction or its degradation products must not cause any negative effect on health. The carrier must also have a proper resistance to protect and deliver the core material [118], [119].

I.5.2.1. Carboxymethyl cellulose and ethyl cellulose

The use of carbohydrates as wall materials is very attractive since they are usually cheap materials, biocompatible, biodegradable, non-toxic and can be used with different encapsulating methods and core materials [120]. Cellulose (Figure 10) is natural water insoluble biopolymer composed of β-linked glucopyranose residues and may have several types of substitution that modify the physical chemical properties of the polymer.

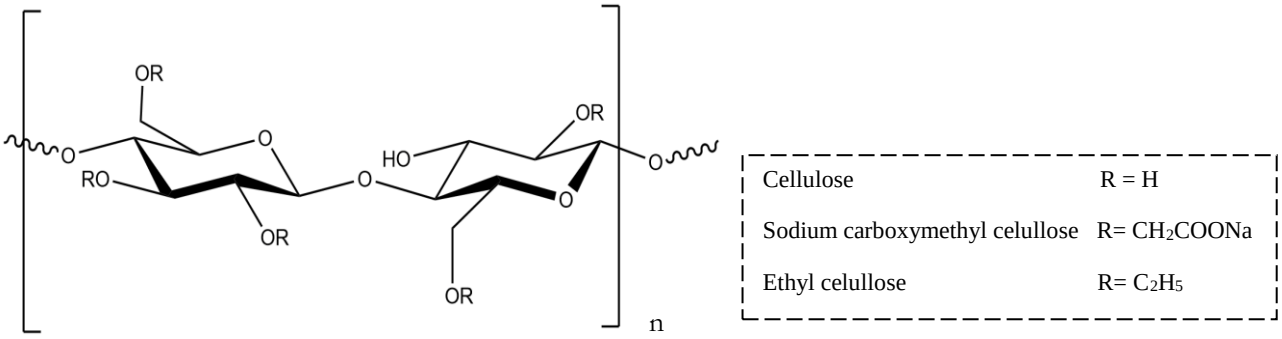


Figure 10 - Chemical structure of cellulose, sodium CMC and EC polymers

Carboxymethyl cellulose (CMC) is a water soluble anionic cellulose derivative with carboxymethyl substitution groups. It can be obtained by the etherification of the polymer with chloroacetic acid. Typically, the commercial form of carboxymethyl cellulose is as white sodium salt and this derivative finds application in a wide range of fields such as food, cosmetic, pharmaceutical, textiles, detergents. Carboxymethyl cellulose is already widely used in cosmetic and personal products as emulsifier, stabilizer, film-former and thickener agent [121]. It is not soluble in organic solvents but it can be dissolved in mixtures of water and water-miscible solvents (e.g. ethanol, methanol). The dissolution rate is proportional to the degree of substitution but it decreases with the molecular weight (i.e. viscosity) [122]. Carboxymethyl cellulose hydrogel swelling behavior is pH and ionic strength dependent due to the presence of electrostatic charges in the polymer network originated from sodium ion and the carboxymethyl group [123]. Films formed using carboxymethyl cellulose have a moderate strength in aqueous solutions although such property is also dependent on the degree of substitution and molecular weight [124], [125]. Carboxymethyl cellulose is frequently used with cross linking agents to improve its performance [123].

Ethyl cellulose (EC) is another derivative of cellulose where some of the hydroxyl groups of cellulose monomer were replaced by ethyl ether groups. It may result from the etherification with ethyl chloride [121]. Its appearance ranges between a white and a light tan powder or granular substance. It is only soluble in organic solvents such as alcohols, ketones, ether and esters which makes its use attractive as encapsulation agent for both water and organic solvents. Although its water insolubility, ethyl cellulose can take up water due to the hydrogen bond potential of the oxygen ethyl group atom with water. In addition, it is biocompatible, odorless, tasteless, non-toxic and non-irritant although it is not biodegradable. Ethyl cellulose is also stable under light, oxygen, heat and it is able to absorb pressure being thus resistant to mechanical stress [126]. Several options of ethyl cellulose are commercially available depending on the degree of substitution and molecular weights (i.e. viscosity). Ethyl cellulose is also used in cosmetic industry as base of cosmetic pastes and to form gels of liquid oils [127]. In fact, ethyl cellulose has been studied to replace petrolatum as cosmetic ingredient [128].

I.5.3. Microparticles controlled release

The term controlled release can be defined as the rate that certain molecules become available under particular conditions or stimulus (e.g. pH, temperature, moisture, enzymes, etc.) [129]. The mechanism by which the core material is released from the microparticles is called release mechanism and it depends on external factors (pH, moisture, enzymes, temperature, mechanical strengths) as well as interactions between the core and shell materials. There are several release mechanisms by which core material can be released: hydrolysis, diffusion through water-filled pores, osmotic pumping, erosion, water absorption/swelling etc. However, they can be summarized in four main processes that are presented in Figure 11.

Diffusion processes and osmotic pumping involve the transport of the core material whereas erosion is caused by polymer disintegration. Assuming water as the fluid where the microparticle is placed, diffusion through water pores is the mostly common mechanism involved in core material release. The fluid penetrates the shell and then dissolves the core that is released through the pores. The release depends on 1) the rate that the fluid penetrates the shell; 2) the rate that the core dissolves in the fluid; 3) the rate that the dissolved core leaks out through pores. In addition, dissolution of the polymer in water may occur at the same time the core material diffuses. Diffusion through the polymer can occur when the core material is small and has a suitable hydrophobicity to diffuse through the polymer. Osmosis can be defined as the transport of core material through water filled pore by a force such osmotic pressure (convection).

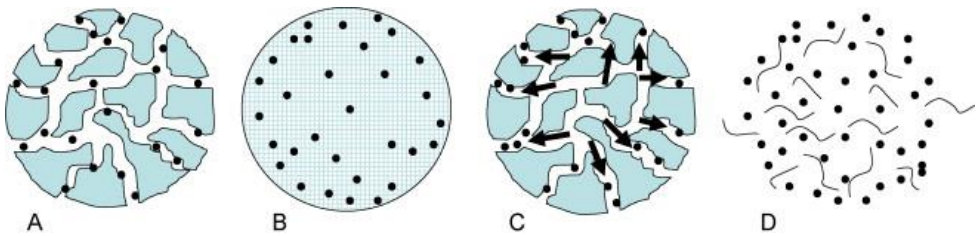


Figure 11 - Release mechanisms: (A) diffusion through water-filled pores, (B) diffusion through the polymer, (C) osmotic pumping and (D) erosion [130]

Osmotic pressure can be generated by the water influx into a non-swelling system since swelling systems are able to compensate the influx of water by swelling and rearrangement of the polymer chains. Erosion is simply the disintegration of the polymer without any transport of the core material. However, erosion can lead to an increase of the core material transportation rate since it creates pores. Although it is possible to know the mechanism by which a substance could be released, it is difficult to assess which one is dominant and in a chain of processes, it may not be clear which one is the rate determining process [80], [130].

For cosmetic application, controlled release is extremely important. Ideally, sensitive active ingredients in cosmetic formulation (e.g. creams) should be entrapped and protected while the product is not applied on the skin by the consumer. At the moment of application, the ingredient should be released. On the other hand, if the encapsulated ingredient is to be used as preservative in cosmetic formulation, different considerations about the release profile should be taken into account. A controlled release should be observed to keep the stability of the cosmetic cream. Factors like temperature, mechanical energy, or enzymes could be responsible for the release of the core material.

Core material release profile is frequently bi-phasic or tri-phasic. In a traditional tri-phasic release, phase I is generally designated as a burst release and it has been attributed to non-encapsulated core molecules on the surface or close to the surface easily accessible. The formation of cracks and the fragmentation of particles can also be responsible for the burst release. Phase II is characterized by a slow release, where the core material diffuses through the pores or the polymer, while degradation or hydration of the shell material occurs. Phase III is a period of faster release due to erosion. Figure 12 presents different illustrative release profiles for poly(lactic-co-glycolic acid) (PLGA) as wall material. [130]. The controlled released assay should be performed in the same matrix and conditions where the microcapsules are going to be incorporated.

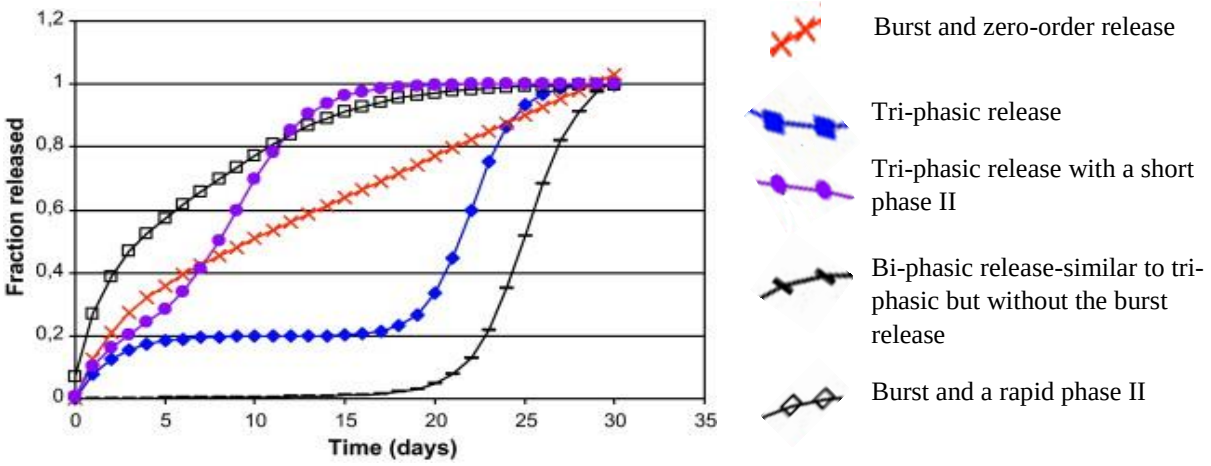


Figure 12 - Release profiles consisting of different phases for PLGA capsules [130].

II. State of the art

The use of delivery systems represents a great interest in the cosmetic industry. Some of the ingredients present in cosmetic formulations, mostly the ones that are responsible for the key features of the product, are only able to play their specific activity when they are properly associated with the right delivery system. Delivery systems must protect and stabilize the ingredient in the formulation, delivery it to the skin, cause adhesion or enhance its penetration in order to accomplish the desired and the best performance. Furthermore, the cosmetic formulation must also be appealing to the consumer regarding the color, spreadability, texture, stability over time. All these factors have been contributing to the increasing demand and development of new competitive delivery systems.

In this chapter some of the research articles from Table B and Table C in appendix 2 will be grouped, briefly described, discussed and compared. Table B and Table C from appendix 2 represents a bibliographic collection with cosmetic relevance, focused mainly in lipid vesicles, lipid nanoparticles and polymeric nano/microparticles carrying, all of them, natural ingredients. Only published articles from the last ten years were considered and the main focus of research was to find microencapsulation bibliography using spray drying method and pure antioxidant ingredients (mainly rosmarinic acid). This chapter was organized by selecting and describing some of the 52 research articles collected in order to provide the reader with the maximum range of studies performed in the cosmetic delivery systems field. In addition, published articles with relevance to the theme of this thesis were also presented. The selected studies were grouped in categories which were created considering not only some of the most common tests to develop a delivery system but also, the ingredients, the methods and tests performed in this work.

A total of 52 research articles were collected however, some of the articles studied more than one type of delivery system. Therefore, Figure 13 was designed considering the 55 delivery systems present in Table B and Table C in appendix 2. It represents the percentage of each type of delivery system studied in all 52 research articles. The different types of vesicles (transfersomes, ethosomes and niosomes) were grouped together to obtain a representative share. The same consideration was made for SLN and NLC. Other delivery systems comprise the combination of SLN, NLC, liposomes with polymeric nano/microparticles.

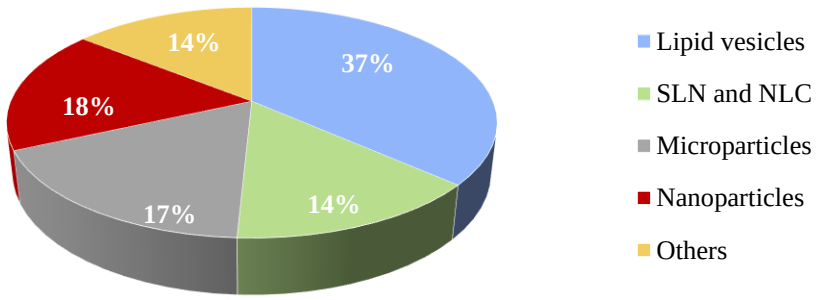


Figure 13 - Distribution of each type of delivery systems (last 10 years) from Table B and Table C from appendix 2

Some general and initial conclusions may be drawn from Figure 13 and the bibliography collection. Vesicular delivery systems were easily found in literature for cosmetic application. Liposomes, transfersomes and ethosomes represented a class of phospholipid vesicles that were easily incorporated in cosmetic formulations such as gels or creams presenting positive results in active ingredient delivering [131]. Niosomes are vesicles composed of biocompatible surfactants that are characterized by an improved elasticity [132]. Surprisingly, not so many studies were easily found about SLN and NLC. According to literature reviews, these technologies have a great potential for cosmetic application. However, only 14% of the 55 delivery systems studied in the collected 52 research articles, claim the use of NLC and/or SLN. Nevertheless, it is important to note that the main focus of research was microencapsulation which can justify the lack of SLN and NLC bibliography. Polymeric micro and nanoparticles together compose 35% of the articles that were found. The use of these technologies was most associated to controlled release of the active ingredient, stability and toxicologist studies of the delivery system [133–135]. Other delivery systems were found more frequently than expected and they showed positive results [102], [136], [137].

II.1. Delivery system ingredients

Rosmarinic acid was the chosen antioxidant to be encapsulated in this work due to its benefic properties for human health reported in chapter I. Therefore, the research was mainly aimed to find delivery systems with this core material. Although its interest for cosmetic industry, only a few studies regarding the incorporation of rosmarinic acid in delivery systems for cosmetic purpose were found. Kim et al. reported the microencapsulation of rosmarinic acid in poly(ε-caprolactone) spheres. Emulsion solvent evaporation method was used and ionic and non-ionic surfactants were tested. Some crucial factors to determine the characteristics of the final product are the encapsulation method and reagents. The zwitterionic surfactant (Cocamidopropyl betaine) led to the smallest particles and with highest entrapment efficiency [56]. Probably the repulsion due to the presence of charged surfactants avoided the aggregation of the particles leading to smaller particles. Interactions between the reagents and the delivery system ingredients should also be taken into account. Long term stability of a cream formulation containing microparticles and free rosmarinic acid was also assessed for 8 weeks. Results showed that the degradation of rosmarinic acid in cream formulation was more pronounced in its free form than when it was encapsulated. The encapsulation of rosmarinic acid in poly(rutin) nanoparticles was reported by Sahiner. Rutin is a polyphenol with antioxidant activity commonly used as active ingredient. However, in this study it was explored as delivery system of rosmarinic acid. The release of rosmarinic acid in PBS, pH 7.4, lasted for 6 days with an almost linear release profile releasing 85% of the loaded antioxidant [138]. Niosomes were also used to carry rosmarinic acid in an anti-acne gel. The release profile from a niossomal gel in PBS, pH 5.5, also revealed an almost linear release in the first 20 hours which may be useful when a constant release rate is required [139]. Usually, the conditions where the release test is performed must be the as similar as possible to the final application. In this way, Budhiraja et al. niosomes preparation may present more reliable results since it was tested at skin pH and after incorporated in a simple

cosmetic formulation. In addition, the incorporation in the hydrogel also slowed down the release of rosmarinic acid from niosomes.

On the other hand, resveratrol and quercetin are extensively explored for cosmetic application. Resveratrol studies represent around 35% of the total active ingredients that were found. This antioxidant was successfully encapsulated in niosomes, liposomes, SLN, NLC, lipid core micro and nanoparticles[136], [137], [140–145]. The protection of the active ingredient is only possible if the delivery system remains stable however, on the other hand, a delivery is only useful if it is able to protect the active ingredient. Detoni et al. encapsulated resveratrol in liposomes, polymeric nanocapsules and NLC. Regarding the photostability of resveratrol, the association of the antioxidant with liposomes resulted in the best combination while with NLC presented worst results. However, concerning the stability of the carrier systems, liposomes revealed a decrease in their size, while NLC size remained constant [144]. These contradictory results regarding the stability of the delivery system and the active ingredient evidence some of the critical aspects of choosing the most suitable delivery system.

During research the encapsulating agent was not one of the main focus since its choice is most of the times by trial and error. Therefore, no studies were found using only ethyl cellulose or carboxymethyl cellulose but the combination of ethyl cellulose with methylcellulose.

II.2. Microparticles and preparation methods - Spray drying and solvent evaporation

Since microencapsulation using spray drying and solvent evaporation was used in this work and the selection of encapsulation method is an important decision, several microencapsulation studies are now presented. Microencapsulation studies regarding specifically cosmetic application were not found so easily as other delivery systems, since most of the microencapsulation research articles claimed food application. Regarding microencapsulation publications, most of the studies presented in Table B and Table C from appendix 2 are related to conventional polymeric microparticles but lipid microparticles (coated or not with polymeric material) were found as well. Concerning the active ingredient, 57% of the 14 microencapsulation studies used a pure antioxidant compound while 35% encapsulated extracts and only one study encapsulated an essential oil.

Taking into account that spray drying is the mostly used technique according to literature reviews, it was expected to find more cases where this technique was used. However, only three authors claimed the use of spray drying being emulsion based techniques more often applied. Green coffee oil was microencapsulated to test its sun protection and antioxidant activities after spray drying by Carvalho et al. Emulsions were stabilized by lecithin and chitosan through electrostatic layer-by-layer deposition technique. The use of corn syrup as wall material led to higher sun protection factors than the control solution however, this wall material could have interfered with the measurements. On the other hand, the use of Hi-Cap 100 as wall material led to an improved oxidative stability. When lecithin-chitosan were used in microparticles preparation, a better

barrier against oxidation was formed [146]. Nosari et al. encapsulate green coffee oil as well by spray drying using Arabic gum. Spray dried powder was characterized and oxidative activity of the microencapsulated green coffee oil was tested using castor oil degradation. Results showed that green coffee oil had a higher antioxidant activity then α- tocopherol. Microencapsulated systems provided a better stability and longer release of green coffee oils which gave a prolonged protection to the castor oil than the free extract [147]. The encapsulation of vitamin E by spray drying using chitosan as wall material was performed by Yenilmez et al. Vitamin E loaded microspheres were tested *in vitro* and the results showed satisfactory antioxidant activity and a controlled release. A sustained release from a formulation was observed during 6 h where 89.1% of the vitamin E was liberated. A burst release phenomena was verified in the first 5 minutes which can be attributed to the presence of antioxidant in the microparticles surface. *In vivo* tests were performed in human volunteers to test the efficacy of cream formulations by detecting modification in skin pH, sebum content, skin roughness, skin moisture and elasticity. No changes in pH or sebum content were observed while there was an increase in skin moisture and elasticity and a decrease in roughness [148].

The decision of using solvent evaporation encapsulation method in this work was made after the bibliography collection. Therefore, the research was not so focused in this method and consequently, only a few studies are present in Table B and Table C from appendix 2. One critical aspect of solvent evaporation method is the separation of solid microparticles from the aqueous phase. Kim et al. and Chaiyasat et al. used centrifugation to extract the microparticles while Barroso et al. used filtration [56], [149], [150]. Another critical aspect is the ratio between the encapsulating agent and the core substance. Barroso et al. studied the influence of the polymer molecular weight (poly(l-lactic acid) - PLLA) and its ratio comparing to the core material (vitamin E). Results evidenced that for low molecular weight PLLA, microcapsules were not formed if the ratio of antioxidant:polymer was under 1:25. On the other hand, if a higher molecular weight polymer was used, a ratio of 3:1 was optimum for microparticles formation [149].

After considering the results of the studies just described in this section, it was expected that the microencapsulation of rosmarinic acid in this work would not affect its good antioxidant activity significantly. Moreover, it was considered that the comparison between spray drying method and an emulsion based method would be interesting, and could help to understand why the last one is so used.

II.3. Controlled release studies

Controlled release studies are probably one of the most important tests to perform when a delivery system is being developed. Considering Table B and Table C research articles in appendix 2, this kind of study has been mostly associated to micro particulate systems. Chitosan was extensively used as wall material in the majority of the research articles found. Hui et al. investigated the encapsulation of Cortex Moutan, a gallic acid rich extract, in two wall materials: chitosan and chitosan/sodium alginate. Both studies presented parabola release profile. Chitosan/sodium alginate microparticle showed a linear relationship in the initial 48 h for the two tested pH values (5 and 5.4) with a faster release rate in the lowest pH value [151]. In contrast to the

previous study, chitosan microparticles release rate was not affected by the pH value in the first 45 h. However, after that, the release rate in pH 5.0 is slightly higher than in pH 5.4 [152]. The results of both studies may allow to conclude that little differences in pH values may have an important impact in the release profile of gallic acid especially, when chitosan/sodium alginate is considered. Vitamin C was also encapsulated using chitosan and glutaraldehyde as cross-linking agent. The release profile showed bi-phasic pattern as well. It also demonstrated an increase in the release rate when higher amounts of core content were encapsulated and less amount of cross linking agent was used [135]. Lam et al. investigated the release of phyllanthin from chitosan microparticles in phosphate (pH 7.4) medium. Approximately 20% of the drug was released in the first 24 h, while in the next 72 h another 25% of the drug was slowly released. After 120 h, more than 60% of phyllanthin had been released [153]. Comparing this last results to the studies of Hui et al. with chitosan, higher pH values may have contributed to the slower release of the drug. Martins et al. encapsulated thyme oil in polylactic acid microcapsule and assessed its release through the quantification of several components such thymol. Results exhibited a faster release in the first hour and an almost constant release in the next days. Data also indicated a faster release of thyme oil polar compounds than the non-polar ones [154].

The release profile of genistein was compared by Kang et al. using conventional liposomes and transferosomes over 24 h. Results did not demonstrate to be significant different with a liberation of around 50% of encapsulated genistein at the end of the experiment [155].

Teskac et al. assessed the release profile of resveratrol from SLN. The resulting plot allowed the authors to propose that 15% of resveratrol was not bound to the SLN. After the first 15 minutes, around 75% of resveratrol, that was suggested to be entrapped or adsorbed on the shell, was released slowly. The remaining resveratrol, incorporated in the SLN core, was gradually released during the next hours [156]. The release from NLC systems were also evaluated. Okonogi et al. studied the release of lycopene using mineral oil in the receptor phase. The graphic suggested a bi-phasic release profile with a fast release at the initial stage (first 6 h) followed by a prolonged release phase for the next 18 h. It was believed that the release from the carrier was linked to the loading of lycopene in formulations and its solubility in the release medium [157]. Kamel et al. evaluated the *in vitro* release of rutin from NLC cream formulation. Results showed a bi-phasic release with a fast release rate in the first 6 h with a liberation of 65% to 85% of rutin depending on the cream formulation. At the end of the experiment (24 h), 75% to 90% of antioxidant had been released [158].

Lipid microparticles entrapping quercetin were also studied. Authors claimed that the release profile did not evidence a burst effect phenomenon [159]. Friedrich et al. studied the combination of resveratrol and curcumin in lipid core nanoparticles surrounded by poly(ε-caprolactone). The release profiles of curcumin did not present many differences when it was co-encapsulated with resveratrol or alone. On the other hand, the release profiles of resveratrol differed when it was encapsulated alone or in combination with curcumin. No differences in the release of resveratrol were observed until 6 h but after that, resveratrol release from the microcapsule with both antioxidants was significantly higher [140].

After taking into account the results of the studies described above in this section, it was expected that rosmarinic acid release in this work would have a bi-phasic profile. However, it would be difficult to predict the release rate.

II.4. Skin permeation and penetration

Considering the 52 research articles collected, a total of 25 studies investigated the ability of the active ingredient to cross or deposit along the skin layers. To compile such information Figure 14 and Figure 15 were designed. Figure 14 presents the percentage of each type of study (i.e. transdermal flux, skin distribution or both studies) performed in the 25 articles. Transdermal flux studies are used to evaluate the ability of the active ingredient to cross all the skin layers while skin distribution evaluates the amount of the active ingredient present in each skin layer. Both types of skin studies were performed typically using Franz diffusion cells and mice, human or pig skin in the experiments. Figure 15 was developed considering all the delivery systems studied in the publications that had assessed the skin distribution of an active ingredient. This type of study was performed for a total of 30 delivery systems. It was only considered that the active ingredient was present in a certain skin layer (i.e. stratum corneum, epidermis or derma) if the authors explicitly said so, otherwise, it was classified as not defined.

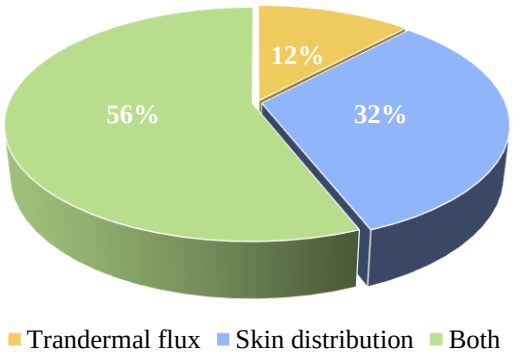


Figure 14 - Distribution of type of skin tests performed

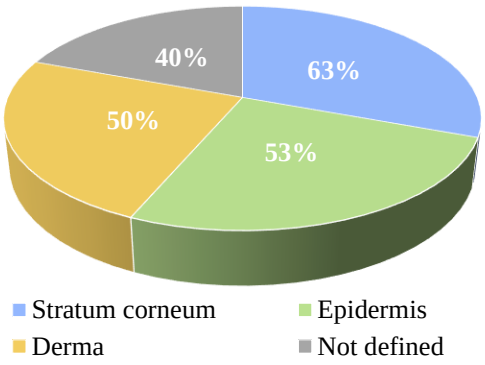


Figure 15 - Skin deposition within the skin layers

Regarding Figure 14, 12% of those studies only broach the transdermal flux of the active ingredient across the skin layers while 32% only studied the presence of the active ingredient in the different layers of the skin. Both types of skin studies were performed by 56% of the researchers. Only one study of micro encapsulation was found to have skin interaction studies. This kind of research was more associated to vesicular and nano particulate systems. Considering the studies that explored the distribution of the active ingredient through skin layers (Figure 15), 63% explicitly reported the presence of active ingredient in the stratum corneum, 53% in the epidermis and 50% in the derma. 40% does not specify where the active ingredient was found but only that it was deposited within the skin layers. These results may evidence the difficulties in reaching the deeper layers of skin. Junyaprasert et al. reported the incorporation of ellagic acid

in niosomal formulations obtained from the mixture of Span 60 and Tween 60. Human skin and Franz diffusion cells were used to perform the skin transdermal flux and distribution assays with phosphate buffer (pH 5.5) and isopropyl alcohol as receptor medium. Regarding the transdermal flux, results evidenced that ellagic acid from niosomal formulations was found in the receptor medium and epidermis while, when solution of ellagic acid was tested, ellagic acid was only found in epidermis but not in the receptor medium. Concerning the distribution of ellagic acid through skin layers, higher amounts of antioxidant were observed in the epidermis layer (including stratum corneum) then in dermis. The solution of ellagic acid was only able to penetrate in stratum corneum in low amounts [160]. These results may prove the potential of niosomes to improve skin passage and its use in cosmetics. Some authors suggest the penetration of active substances through the hair follicle. Kang et al. tested the penetration of genistein loaded elastic liposomes in hair and hairless skin from rat and mouse, respectively. The receptor solution was N,N-diethylnicotinamide in phosphate buffer. A decreasing permeation rate of genistein and skin deposition levels in hairless skin was more evident for elastic niosomes which may suggest that hair follicles can work as reservoir of genistein [155]. Oliveira et al. performed *in vitro* toxicological assays in human keratinocyte cells and *in vivo* skin tolerance to rutin loaded gelatin nanoparticles in cosmetic sun protective formulations. Safety assessments for nano particulate systems were found to be frequent since these particles can interfere in the normal function of the human body and be easily absorbed due to their small size. Results confirmed the safety of the delivery system tested [134]. Some studies also investigated the uptake of the delivery system by human keratinocytes. Moulaoui et. al. tested the uptake of ethosomes and phospholipid vesicles carrying *Fraxinnus angustifolia* extract. Results showed a faster internalization of ethosomes but both types of delivery systems were uptake [161]. Some authors evaluated the efficacy of formulations in order to determine if they are able to fulfill their function when a delivery system is used in the formulation. Lee et al. evaluate the ability of cream formulation with niacimide – flexible liposomes to white the skin in 4 and 8 weeks of application. The amount of melanin was quantified and visual results were also considered. Final results seemed to be satisfactory [162]. Kwon et al. used animals to study anti-wrinkle and depigmentation effect of *P. aviculare* extract loaded liposomes. A reduction in wrinkle formation was achieved for the two types of liposomes tested. Peptide conjugated liposomes evidenced a greater effect in depigmentation [163].

The results of the studies just described in this section suggest that skin penetration future work using rosmarinic acid loaded microparticles may demonstrate higher amounts of antioxidant in the upper skin layers. The tests should be done using Franz diffusion cells and ideally, human skin. Hair follicle may be used as one of the main penetration pathways of the antioxidant.

II.5. Incorporation of delivery systems in formulations

Incorporation in cosmetic formulations could be one of the most critical aspects of developing a new product with a delivery system. Additionally, the release profile and the behavior of the active ingredient can change when they are incorporated in cosmetic formulations or/and when they are associated to a delivery

system. Scalia et al. tested the *in vitro* release of resveratrol- chitosan coated lipid microparticles from cream formulation. At the end of 6 hours, around 50% of resveratrol was released when it was present in a cream formulation in its free form. On the other hand, approximately 30% of resveratrol was released when it was entrapped in the microparticles. The use of resveratrol loaded in chitosan coated lipid microparticles also enhances its *in vivo* penetration in stratum corneum [136]. Two different vehicles (water and cream) were used to test the penetration of free and encapsulated *Garcinia mangostana* Linn extract in porcine ear skin by Tachaprutinun et al. The main results evidenced that cream formulations penetrated deeper into the hair follicles than aqueous formulations. However, a relatively homogeneous distribution on the corneocytes were observed for both aqueous and cream formulation [164].

The manufacturing step of a cosmetic product where a delivery system is incorporated is also important to ensure the stability of the final product. An extract of *Helichrysum stoechas* (L.) Moench phenolic compounds was encapsulated in polycaprolactone diol and incorporated in a base moisturizer. The base moisturizer was prepared by the conventional o/w technique. The lyophilized microparticles had to be dispersed in the oily phase of the moisturizer preparation to avoid agglomeration. Microparticles were observed in the moisturizer base as individualized particles [150]. Quercetin was entrapped in lipid core microparticles which were incorporated in the aqueous phase of a cream formulation. The encapsulation of quercetin prevented its degradation and long term stability tests demonstrated that microencapsulation improved the chemical stability of the antioxidant when it was present in a cream [159].

Several studies reported the characterization of gel formulations in order to find if they are sensorially more attractive and to explore their long time stability. A wide variety of factors may be tested in order to evaluate the formulation e.g. temperature, rheological properties, homogeneity, spreadability, pH. Vyas et al. concluded that the incorporation of caffein loaded liposomes in a 2% carbopol gel had suitable rheological properties to combat cellulite by applying the gel on the skin. Stability studies also suggested that keeping the liposomal gel at low temperatures would minimize the stability problems of liposomes [131]. Manosroi et al. explored the stability of papain loaded niosome gel, with 8% carbopol, regarding color, homogeneity and phase separation during 3 months. No sedimentation, no layer separation and no color changes were observed when stored at different temperatures. Only the gel with free papain became light yellow when stored for 2 months at 45 ºC. Zeta potential was almost the same after stored for 3 months at different temperatures but a decrease in viscosity value was observed [132]. Rigo et al. studied the pH, spreadability, and rheological behavior of a gel formulation after incorporating rice bran oil nanocapsules. All the formulations presented a uniform color and pH values suitable for topical application. Encapsulation turns the gel sensorially more attractive since it did not feel greasy or sticky. All semisolid formulations showed pseudoplastic non-Newtonian behavior [102]. Kaur et al. tested the efficacy of ethosomal, transfersomal and liposomal cream formulations incorporating *C. longa* extract. Cream formulation efficacy tests were performed in human volunteers through the evaluation of skin hydration and sebum content. Results demonstrated that cream best performance was in the order transfersomal > ethosomal > liposomal > free *C. longa* > Empty transfersome > Empty ethosome > Empty

liposome > Base cream. Formulation containing *Curcuma longa* extract can be used as photoprotective creams [165].

The results of the studies presented above may indicate that the release profile of microparticles could be different when incorporated in a cosmetic formulation. In addition, long-term stability, physical-chemical properties, appearance should be assessed as well if the formulation is sensorially attractive, safe and effective.

III. Equipment and reagents

III.1. Reagents

Rosmarinic acid (Ref. 536954-5G) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was obtained from VWR International (Fontenay-sous-Bois, France). Deionized water was obtained in the laboratory using a Merck Millipore Milli-Q water purification equipment (Billerica, MA, USA). Carboxymethyl cellulose was purchased from VWR (Haasrode, Belgium). Ethyl cellulose was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1- Octanol was obtained from VWR (Fontenay-sous-Bois, France). Dichloromethane was obtained from (Merck, Darmstadt, Germany). Polyvinyl alcohol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trolox was obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Applichem (Darmstadt, Germany). Potassium persulfate was obtained from Panreac (Barcelona, Spain) Ethanol was purchased from AGA (Prior Velho, Portugal)

III.2. Equipment

All weight measurements were performed using a Mettler Toldedo AG245 analytical balance (Columbus, OH, USA). pH measurements were performed using a 900 Multiparameter Water Quality Meter (A & E Lab; Guangzhou, China). Dissolution was performed using P. selecta ultrasound (Spain). Rosmarinic acid quantification and antioxidant activity was performed using a Jasco V-530 UV-Vis Spectrophotometer (Easton, USA). Spray drying was performed using a BÜCHI B-290 advanced spray dryer (Flawil, Switzerland). Solvent evaporation particles were dried using vacuum oven BÜCHI GKR-5 (Switzerland). Particle size distribution was measured by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, FL, USA). Particle morphology was evaluated by Scanning Electron Microscopy, (SEM) (Quanta 400 FEG ESEM / EDAX Genesis X4M).

IV. Methods

IV.1. Analytical method for rosmarinic acid quantification

In order to get fast results, in a simple and cheap way, it was decided to use UV-Vis-spectrophotometry instead of High-performance liquid chromatography (HPLC) as analytical method for rosmarinic acid quantification. HPLC analysis could also be performed however, it is known that microparticles can block and damage the column due to accumulation in the system. It was possibly to use UV- spectrophotometry since the samples were composed of only one substance. Otherwise, HPLC must have been used. The absorbance spectrum for rosmarinic acid was performed within a range of 190 to 700 nm. The standard solutions for the standard curve were measured at 324 nm.

IV.1.1. Standard solutions preparation

Two stock solutions in ultrapure water and octanol were prepared. One stock solution of 375 mg/L of rosmarinic acid was prepared in 25 mL of ultrapure water and methanol (50:50 v/v) by weighting 9.8 mg of the antioxidant using an analytical balance. A second stock solution of 490 mg/L of rosmarinic acid was prepared in 10 mL of octanol and sonicated. Then, both stock solutions were stabilized to guarantee homogenization at 4 ºC, during 24 hours, sealed with parafilm and wrapped in aluminum foil. The storage conditions were at - 20 ºC, sealed with parafilm, in ambar flasks to protect from light. Eight standard solutions were prepared from dilution of the stock solutions with concentrations of rosmarinic acid of 1.0, 3.0, 6.0, 9.0, 12, 15, 18, 20 mg/L. All standard solutions were stabilized for 2 hours, sealed with parafilm and wrapped in aluminum foil and then, transferred to ambar flasks.

IV.1.2. Standard curves validation

To guarantee the reliability of the results and to prove that this analytical method is suitable for rosmarinic acid quantification an analytical method validation was performed. The two kind of parameters considered were: quantification parameters (sensitivity, linearity, limit of detection and quantification) and reliability parameters (repeatability, accuracy and intermediate precision).

The standard solution results of UV-Vis-spectrophotometry were processed statistically in order to define the standard curve equation and the correlation coefficient R, using Microsoft excel 2016 software. It was expected to obtain a linear relation between the absorbance values and the concentration of standard solutions since absorbance is directly proportional to concentration of the sample according to Beer-Lambert law. UV- spectrophotometric results were plotted in the vertical axis while concentrations were plotted in horizontal axis. To evaluate and validate the methods at least 5 standard solutions should be included with concentrations in a range factor superior to 10. The correlation coefficient should be at least 0.99 and the

sensitivity of the method is estimated through the line slope. In order to validate the standard curve and subsequently the analytical method the following conditions must be met:

- Analysis of at least 5 different standard solutions concentrations
- Linearity range in a factor superior to 10
- $R^2 \geq 0.99$
- $\frac{S_a}{a} \leq 5\%$
- $b - tS_b < 0 < b + tS_b$

Where a is the regression slope, b the intercept of the regression and S_a and S_b their standard deviations, respectively.

The limit of detection (LOD) is the lowest amount of rosmarinic acid that can be detected in a sample while the limit of quantification (LOQ) is the lowest amount of antioxidant that can be quantified with acceptable accuracy and precision. In UV spectrophotometry, LOD and LOQ can be calculated through the following expressions:

$$LOD = \frac{3 \times S_b}{a} \qquad \text{Equation 1}$$

$$LOQ = \frac{10 \times S_b}{a} \qquad \text{Equation 2}$$

The reliability parameters were assessed using three different standard solutions concentrations: low, intermediate and high concentration. The intermediate precision was evaluated during three days with two measurements of each concentration. The repeatability (or intraday precision) and accuracy were evaluated in one day with six measurements of each concentration. Both intermediate precision and repeatability were calculated through coefficient of variation (%CV) while accuracy was determined through recovery percentage (%R).

$$\boldsymbol{CV}(\%) = \frac{S_y}{\sim y} \qquad \text{Equation 3}$$

$$\boldsymbol{R}(\%) = \frac{RA_{expected} - RA_{obtainded}}{RA_{expected}} \qquad \text{Equation 4}$$

Where ~y is the mean of the absorbance results and S_y its standard deviation. $RA_{expected}$ is theoretical mass value of rosmarinic acid while $RA_{obtained}$ is the mass of rosmarinic acid obtained through the measurements in the spectrophotometer.

IV.2. Microencapsulation methods

IV.2.1. Spray drying

Two solutions containing the wall material, carboxymethyl cellulose and the antioxidant, rosmarinic acid, were prepared to perform spray drying. A solution of 10 g/L of carboxymethyl cellulose was prepared in 250 mL of ultrapure water by weighting 2.5 g of the wall material. The antioxidant solution was prepared by dissolving 50 mg of rosmarinic acid in 50 mL of ultrapure water. Both solutions were prepared 24 h before spray drying but carboxymethyl cellulose solution was left under stirring at room temperature, while rosmarinic acid solution was wrapped in aluminum foil, sealed with parafilm and stored at 4 ºC. Before encapsulation both solutions were mixed together under stirring during 30 minutes at room temperature. The mass ratio between rosmarinic acid and wall material was 1:50 and the antioxidant concentration in the feed solution was around 2%. Spray drying was performed using a BÜCHI B-290 advanced spray dryer (Flawil, Switzerland) with a standard 0.5 mm nozzle. The operating conditions are present in Table 5 and were optimized by Estevinho et al. [166]. Empty CMC microparticles were also prepared by feeding the spray drying only with the polymer solution. The powders were collected and stored in falcon tubes, wrapped in aluminum foil, and stored at 4 °C.

Table 5 - Spray drying operating conditions

Inlet temperature	115 ºC
Outlet temperature	56 ºC
Aspirator	100%
Pump	15%
Nozzle Cleaner	3

IV.2.2. Solvent evaporation - O/W emulsion

A mass of 300 mg of ethyl cellulose was weighted to a 10 mL volumetric flask and it was dissolved in a mixture of dichloromethane and methanol (1:1 v/v) by sonication. Rosmarinic acid was weighted to a flask (100 mg) and the polymer solution was then admixture in the absence of light. A solution of 1.5% (w/w) of poly(vinyl alcohol) (PVA) was prepared in 250 mL of ultrapure water. The dispersion was added slowly to the PVA solution, under stirring (400 rpm), in the dark, to obtain polymer droplets. Then, it was left under the same conditions, for 2 h to promote the evaporation of the solvent and the microparticles hardening. After that, the microparticles were filtered using a G4 filter and washed with ultrapure water. Finally, the microparticles were dried for 24 h, 25 ºC, in a vacuum oven. For this work, in order to obtain enough amount of microparticles, two batches were prepared in the same day, under the same conditions, and then were mixed together. Empty microparticles were also prepared.

IV.3. Characterization of the microparticles

Microparticles were characterized according to their morphology and size distribution. Product yield, encapsulation efficiency and drug loading were also calculated using equations from Table 4 from chapter I.

Microparticles morphology was visualized by a high resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: (Quanta 400 FEG ESEM / EDAX Genesis X4M). Samples were coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment.

SEM analysis was performed at 15 kV, low vacuum, with magnification of 100–10 000 x, for surface structure observation.

Particle size distribution was measured using a Coulter Counter-LS 230 Particle Size Analyzer through laser granulometry technique. A small amount of powder was dispersed in ethanol and then introduced in the equipment. The particles were characterized by volume and number distribution using Laser Diffraction and Polarization Intensity Differential Scattering. The results were obtained as an average of two 60 s runs.

IV.4. Controlled release studies

The controlled release studies were performed in ultrapure water (pH 5.6) and octanol to simulate cosmetic formulations. For spray drying samples, 3 mg of powder was weighted into separate flasks (in duplicate) and then, 4.5 mL of the solvent was added to each flask. The release was performed at room temperature (20-25 ºC), under low stirring and in the absence of light (by wrapping the flask with aluminum foil). Samples were taken every 0, 1, 2, 5, 10, 20, 30, 45 minutes and 1, 2, 4, 6, 24 hours to evaluate the amount of rosmarinic acid released using the UV-Vis-spectrophotometry method. For solvent evaporation method samples, 10 mg of powder was weighted into separate flasks (in duplicate) and then, 3.5 mL of the solvent was added to each flask. The release was performed under the same conditions as spray drying samples. Samples were taken every 0, 2, 7, 15, 30 minutes and 2, 5, 24 hours to evaluate the amount of rosmarinic acid released using the UV-Vis-spectrophotometry method. More spaced time intervals were considered comparing to spray drying due to low amount of microparticles obtained by this method.

IV.5. Antioxidant activity assessment

To estimate the antioxidant activity of rosmarinic acid after encapsulation, the ABTS radical scavenging assay was performed. Two aqueous stock solutions containing 7.4 mM of ABTS and 2.6 mM of potassium persulfate were prepared in 5 mL volumetric flasks. Then, a working solution was prepared by mixing the two stock solutions and by allowing them to react for 12 h, at room temperature (20- 25 ºC) in the dark. After that, the working solution (starting with 1 mL) was diluted in 60 mL of methanol until an absorbance of 1.10 ± 0.02 AU at 734 nm. To perform the antioxidant activity assay, 150 µL of each sample was mixed with 2850 µL of

the prepared solution (1:20 v/v) and it was allowed to react for 2 h in the dark. Samples consisted in rosmarinic acid spray drying and o/w solvent evaporation microparticles at maximum release and free rosmarinic acid at the same concentrations. The amount of ABTS radical still present in each sample, which corresponds to antioxidant activity, was assessed by UV-Vis spectrophotometer at 734 nm. A standard curve was prepared using trolox, an analogue of vitamin E. To do so, a stock solution of 500 µM was prepared in methanol, stabilized to guarantee homogenization at 4 ºC, during 24 hours, sealed with parafilm and wrapped in aluminum foil. Seven standard solutions were prepared from dilution of stock solutions with concentrations of trolox of 10, 50, 100, 150, 200, 300, 380 µM. All standard solutions were stabilized for 2 hours, sealed with parafilm and wrapped in aluminum foil and then, transferred to ambar flasks.

IV.6. Waste treatment

All the residues of this work were collected in closed containers, labelled for further treatment by the Environmental Management System of FEUP – EcoFEUP.

V. Results and discussion

In this chapter the results regarding the analytical methods validation, rosmarinic acid microparticles characterization and controlled release studies in water and octanol will be presented.

Firstly, analytical methods validation was performed to ensure a robust and reliable way to quantify the rosmarinic acid release from microparticles. The results are discussed in section 1 of this chapter.

Subsequently both rosmarinic acid microparticles prepared by spray drying and O/W solvent evaporation were characterized regarding its morphology, size, shape and drug loading. Encapsulation efficiency and product yield of the process are also present in section 2 of this chapter.

Section 3 of this chapter reports the results regarding the controlled release studies of both types of microparticles prepared. The release medium was water and octanol to simulate the conditions of a cosmetic product. The release was performed over a period of 24 h and UV-spectrophotometry analytical method was used to quantify the amount of rosmarinic acid released.

Finally, section 4 describes the antioxidant activity of rosmarinic acid after encapsulated. ABTS radical scavenging assay was used and the results were expressed in μM trolox equivalents (TE). To quantify the amount of ABTS radical, UV-Vis-spectrophotometry method was also used.

V.1. Analytical method validation

In order to perform the quantification of rosmarinic acid in water and octanol by UV-Vis-spectrophotometry analysis after release from microparticles, two standard curves were plotted. Eight standard solution, with concentrations from 1.0 mg/L to 20 mg/L were prepared from a stock solution. The analysis was performed at 324 nm using distilled water or octanol as reference.

The resulting standard curve equation in water was Abs = 0.059 ± 0.003[RA] + 0.02 ± 0.03 where the slope (0.059 L.AU/mg) represents the method sensitivity. Octanol standard curve equation was Abs = 0.0457 ± 0.0009 [RA] - 0.002 ± 0.008. Both plots are presented in appendix 3. The parameters that allow the analytical method validation are listed in Table 6. All the five conditions for UV-Vis-spectrophotometry analytical method validation were verified and so the analytical method was validated for both water and octanol. The correlation coefficient value was very satisfactory and a confidence interval of 95% was considered for the slope and intercept regression.

Table 6 - Linearity conditions for the validation of the UV-Vis-spectrophotometry standard curves

Parameters	Water standard curve	Octanol standard curve
At least 5 different standard concentrations	6	6
Linearity range factor >10	15	18
$R \geq 0.995$	0.999	0.999
$S_a/a \leq 5\%$	2	1
$b-tsb < 0 < b+tsb$	-0.01 < 0 < 0.04	-0.01 < 0 < 0.007

Table 7 presents the limit of quantification (LOQ) and the limit of detection (LOD) of rosmarinic acid in both release mediums. As predictable, the LOD was lower than the LOQ but both values are satisfactorily low. However, it is important to note that for water, LOQ was higher than the lowest standard solution concentration considered in the calibration.

Table 7 - Limit of quantification and limit of detection of the UV-Vis-spectrophotometry water and octanol standard curves

	Water standard curve	**Octanol standard curve**
Limit of quantification (mg/L)	1.66	0.700
Limit of detection (mg/L)	0.497	0.210

The intermediate precision and the repeatability allows to understand the results variation of the same sample in long period of time or in a short period of time, respectively. On the other hand, accuracy evaluates the accordance between the measurement and the real value. Both intermediate precision and repeatability are calculated through coefficient of variation (%CV) while accuracy is by recovery percentage (%R). Table 8 presents the results for three distinct concentrations.

Table 8- Intermediate precision, repeatability, accuracy for rosmarinic acid quantification method in water and octanol

	Concentration of RA (mg/L)	**Intermediate Precision (%CV)**	**Repeatability (%CV)**	**Accuracy (%R)**
Water	3	5.6	0.4339	99.58
	9	2.5	0.06912	94.10
	15	1.8	0.112	98.2
Octanol	3	7.2	0.755	97.2
	9	1.83	0.290	97.2
	18	1.03	0.292	99.7

Either the coefficient of variation of intermediate precision and repeatability should be less than 5%. For all concentrations this was verified except for the intermediate precision and repeatability at 3 mg/L of rosmarinic acid in water and octanol, respectively. These results are usually verified for lower concentrations since the method is more sensitive to low concentrations than higher. Regarding the accuracy, it should be as maximum as possible close to 100% and so, results are satisfactory for all the three concentrations in both dissolution mediums. Although the high %CV for the intermediate precision and repeatability of the solution with 3 mg/L of rosmarinic acid in water and octanol, respectively, it was possible to conclude that no significant variations were observed and the analytical method reproduces reliable results.

V.2. Microparticles characterization

V.2.1. Product yield, encapsulation efficiency, drug loading

Product yield is an important aspect of the encapsulation process since it reveals the amount of powder that it possible to recover comparing to the initial raw material. Encapsulation efficiency measures the amount of antioxidant that was encapsulated comparing to the initial solution. On the other hand, drug loading measures the amount of antioxidant in the microparticle. Figures 16 and 17 present the product yield and encapsulation efficiency of ethyl cellulose loaded rosmarinic acid (EC-RA) microparticles and carboxymethyl cellulose loaded rosmarinic acid (CMC-RA) microparticles prepared by solvent evaporation method and spray drying, respectively. Table 9 presents the experimental drug loading for both microparticles as well the theoretical drug loading value considering no losses and a constant ratio between the antioxidant and the polymer during encapsulation process.

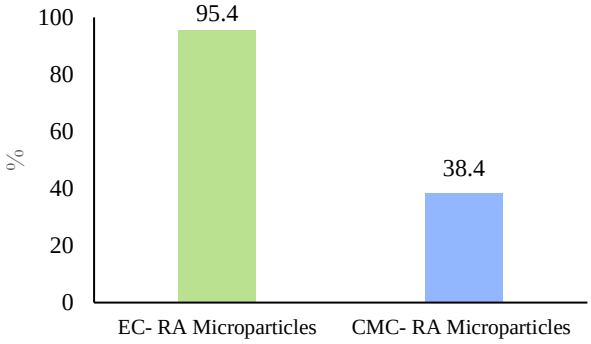


Figure 16 - Product yield of EC-RA and CMC-RA microparticles prepared by o/w solvent evaporation and spray drying, respectively

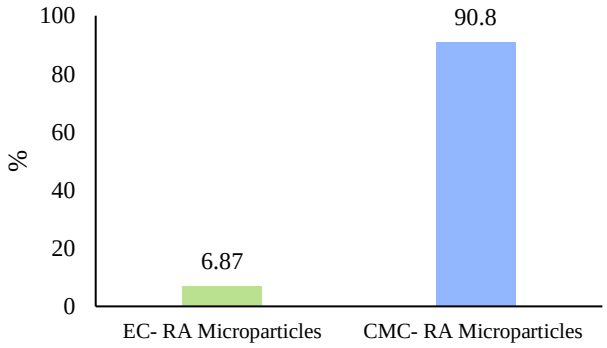


Figure 17 - Encapsulation efficiency of EC-RA and CMC-RA microparticles prepared by o/w solvent evaporation and spray drying, respectively

Table 9 - Experimental and theoretical drug loading of CMC-RA and EC-RA microparticles

	Experimental drug loading (%)	**Theoretical drug loading (%)**
CMC-RA microparticles	1.78	1.96
EC-RA microparticles	1.80	25.0

As it was reported by Giunchedi et al. spray drying is characterized by a higher encapsulating efficiency than solvent evaporation. In contrast, typically solvent evaporation method has higher product yield and low encapsulation efficiency [167]. These tendencies were both observed for EC-RA microparticles and CMC-RA microparticles. Spray drying low product yield may be explained by the small amount of raw materials when compared with the equipment dimensions and by the particles suction through the vacuum filter due to their small size. In addition, since the powder is too thin, it remains stuck to the equipment walls being difficult to collect. Regarding the drug loading, both types of microparticles presented similar contents of antioxidant. For

CMC-RA particles the drug loading is the same as the theoretically expected which confirms one of the basic spray drying assumptions that the ratio between the antioxidant and the wall material remains constant during the process. On the other hand, for EC-RA microparticles, the drug loading is far below the theoretically expected. These results indicate and confirm that the ratio between the antioxidant and the wall material cannot be considered constant during the process and a considerable amount of antioxidant escaped to the aqueous phase. Encapsulating efficiency may be controlled by the solidification rate of the microparticles. A fast solidification usually leads to high encapsulation efficiencies which can justify the big difference between spray dried and solvent evaporated microparticles [168]. In o/w solvent evaporation, there is also a shrinkage during particle formation and the antioxidant can be drained out of the particle [169].

V.2.2. Particles morphology

Both CMC and EC microparticles loaded with rosmarinic acid were observed using scanning electron microscopy. CMC-RA microparticles prepared by spray drying (Figure 18) presented a smooth surface and a spherical regular shape. Apparently, the diameter ranged between a few micrometers and the nanoscale which may justify why particles tended to agglomerate.

Casanova et al. prepared chitosan and modified chitosan microparticles loading rosmarinic acid by spray drying under the same conditions. Modified chitosan microparticles presented a similar shape and surface comparing to CMC microparticles. Chitosan microparticles presented some indentations that were not observed in CMC-RA microparticles [170].

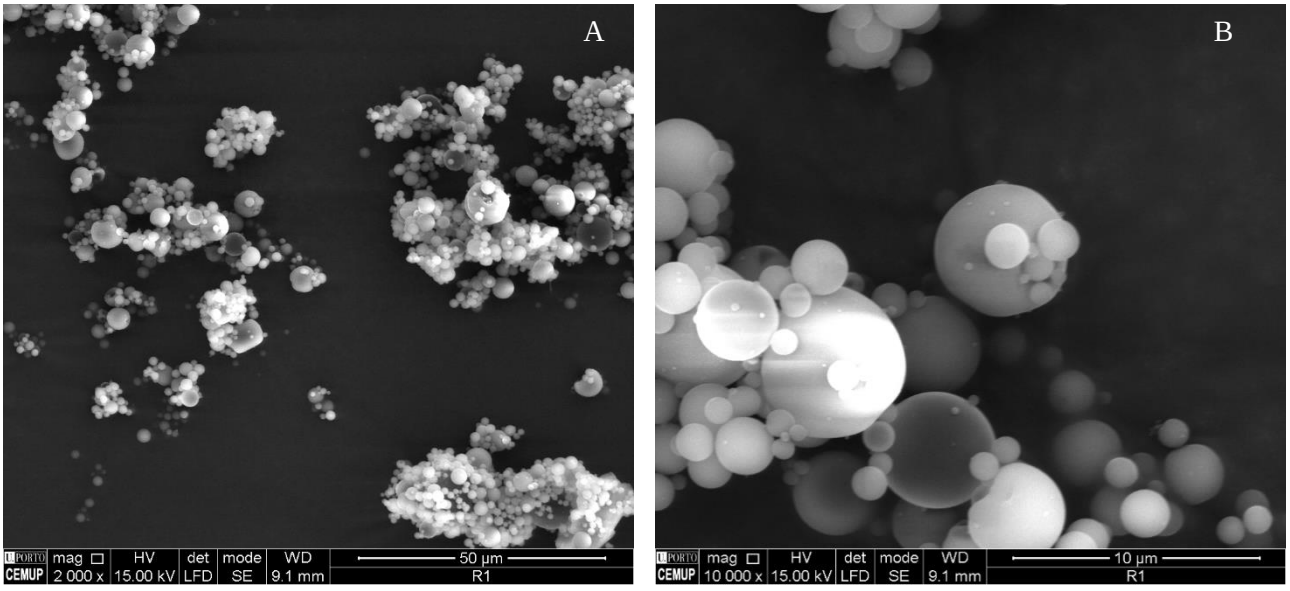


Figure 18 - SEM image of CMC-RA microparticles prepared by spray drying

EC-RA microparticles prepared by o/w solvent evaporation are presented in Figure 20. Microparticles presented an irregular shape as well as some roughness on the surface. The microparticles seemed to present a porous interior as it is suggested by Figure 20. B. After microparticles drying it was necessary to disrupt the formed agglomerate which may have cracked some of the microparticles allowing to visualize their inner structure. The presented results regarding the EC-RA microparticles were not completely unexpected. On one hand, the macroscopy appearance of the particles seemed to be irregular as it was confirmed by SEM exam. However, on the other hand, some authors report a spherical regular shape for microparticles prepared by this method [171], [172]. Surface characteristics may range according to the preparation conditions. Probably, the formation of these structures may be related to the fact that the solvent was not completely removed from the extraction phase exercising a plasticizing effect on the microparticles during drying [173].

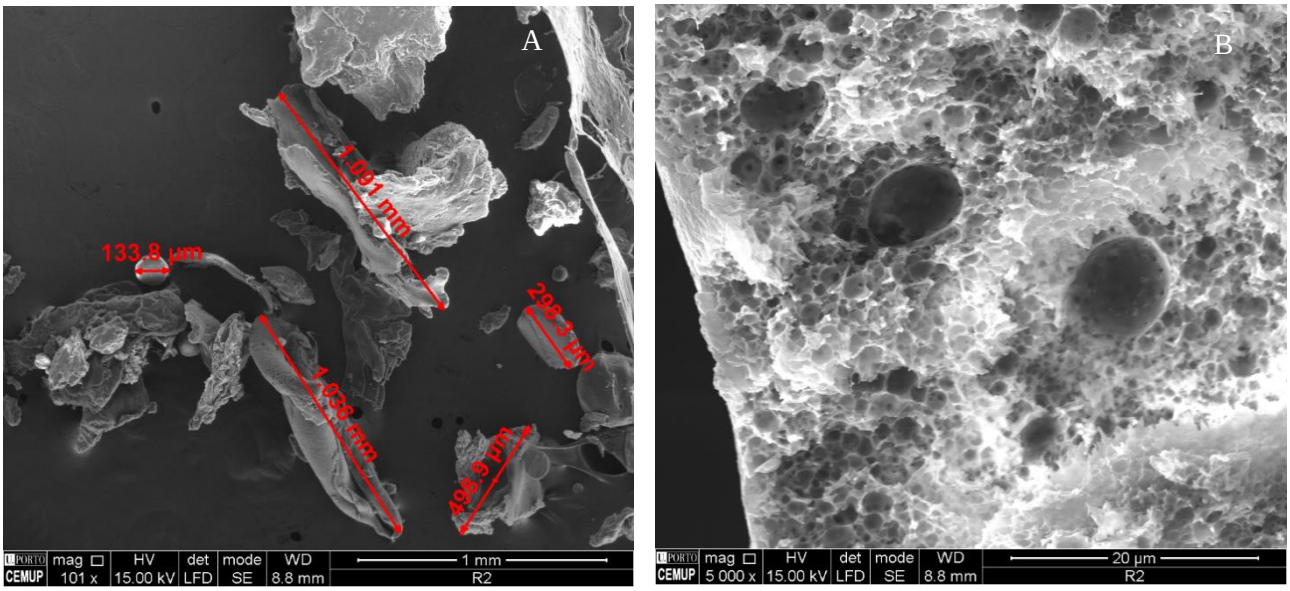


Figure 19 - SEM image of EC-RA microparticles prepared by o/w solvent evaporation

V.2.3. Size distribution analyses

Particle size distribution analysis could be helpful to understand the behavior of the microparticles regarding the controlled release of rosmarinic acid, the loading capacity and infer microparticles stability. In addition, it could help to predict the distribution of the particles among skin layers and to determine the mechanism by which rosmarinic acid is delivered to the skin. Figure 20 represents the number and volume size distribution for CMC microparticles (i.e. obtained by spray drying) while Figure 21 represents the number and volume size distribution for CMC microparticles loaded with rosmarinic acid. CMC empty microparticles were analyzed to assess the influence of rosmarinic acid encapsulation in size distribution. Table 10 presents the number and volume mean size for both particles. The test was also performed for EC microparticles (i.e. obtained by O/W solvent evaporation) but it was not possible to analyze the sample due to the low amount of powder.

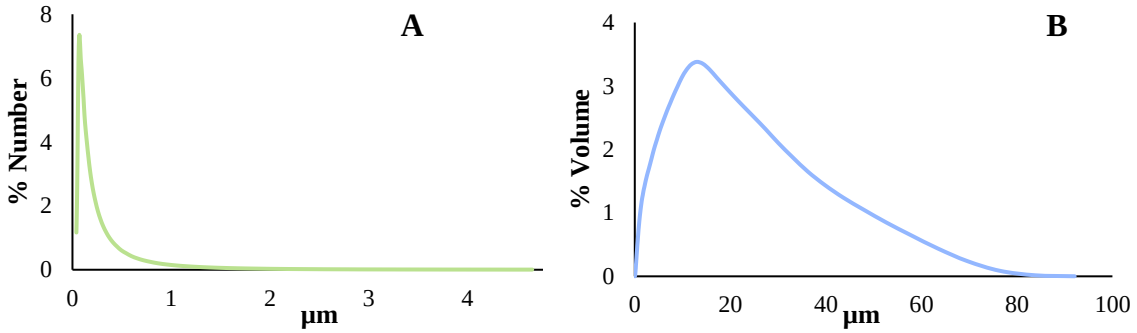


Figure 20 - Number (A) and Volume (B) distribution of CMC microparticles prepared by spray drying

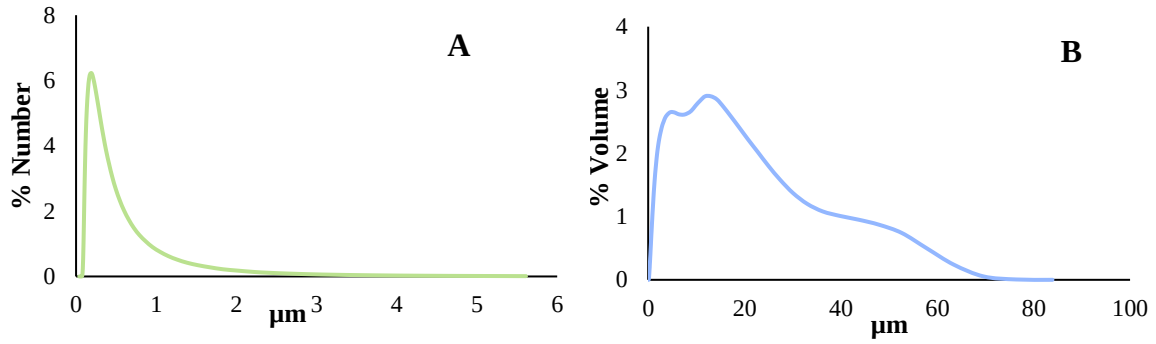


Figure 21 - Number (A) and Volume (B) distribution of CMC loaded RA microparticles prepared by spray drying

Table 10 - Number and Volume average size for CMC and CMC-RA microparticles

	Number mean size (µm)	**Volume mean size (µm)**
CMC microparticles	0.156	13.49
CMC-RA microparticles	0.358	10.90

According to data of Figure 20 and Table 10, considering number distribution, 7.4% of the microparticles presented a diameter of 0.07 µm and a mean size of 0.156 µm. In addition, 90% of the particles were smaller than 0.287 µm. In terms of volume, particles with 12.99 µm were the more representative and the mean size was 13.49 µm. Furthermore, 90% of the volume was represented by particles smaller than 32.36 µm. Considering CMC-RA microparticles and their number distribution, 6.2% of the sample was composed of particles of 0.195 µm and the sample presented a mean size of 0.358 µm. Additionally, 90% of the particles were smaller than 0.673 µm. Particles of 11.83 µm and 12.99 µm were again the more representative in terms of volume but the mean size was 10.90 µm. Moreover, 90% of the volumes was represented by particles smaller than 26.85 µm. These results may demonstrate that the biggest size share was for nanoparticles but they represented a small fraction of the total sample volume. In addition, these results also suggest a size heterogeneity of the powder or agglomeration of the particles.

V.3. Controlled release studies

The release of rosmarinic acid from microparticles was performed in water (pH 5.5) and octanol to simulate cosmetic formulations and evaluate the release profile of rosmarinic acid microparticles when they are incorporated in cosmetic products. The release was assessed during 24 h. Spray drying often requires the use of hydrophilic polymers as wall materials while solvent evaporation typically, requires a polymer soluble in a water immiscible organic solvent. These conditions made difficult to use the same polymer for both encapsulation methods.

The release profile of CMC rosmarinic acid loaded microparticles prepared by spray drying, in water (Figure 22), can be characterized by a sustained release during the first 4 hours where 50 % of the encapsulated rosmarinic acid was released in the first 10 minutes. After the 4 hours, all the rosmarinic acid was released from the microparticles with a complete dissolution of CMC in water. No burst release phenomenon was observed. The final amount of all the rosmarinic acid present in solution measured by UV- spectrometry was only around 10% different from the one predicted theoretically which confirms the basic assumption of spray drying method that the ratio between wall material and core material is kept constant during spray drying. The release profile of CMC rosmarinic acid loaded microparticles in octanol was not possible to assess. CMC is not soluble in organic solvents and when added to octanol the particles became dispersed which made impossible to take a homogeneous sample. In addition, the release of the encapsulated drug is most of the times associated to swelling phenomenon in CMC particles however, this does not occur in organic solvents when the polymer is not soluble. Therefore, the release of rosmarinic acid was not possible to assess due to the small amounts of rosmarinic acid released in the first hours and the heterogeneous samples. Furthermore, when the release time was prolonged the mixture became turbid which made impossible to use UV-Vis spectrophotometry (Figure 23).

The release profile of EC rosmarinic acid loaded particles, prepared by solvent evaporation is presented in Figure 24 for both water and octanol release mediums. A fast consideration can be immediately taken by observing the plot: the release in octanol was faster than in water. This result may be explained by the solubility of EC since it is soluble in organic solvents but not in water. As well as CMC, the release of the encapsulated drug is mostly related to the swelling mechanism but in EC, this is only possible with an organic solvent. The dissolution of the polymer led to a faster release of rosmarinic acid. Unlike CMC that is not able to stablish any interaction with organic solvents, EC can interact with water molecules which promotes the antioxidant release by water up take. However, the release rate is slower and the complete release of the antioxidant does not occur in 24 h since there is no dissolution of the polymer. Therefore, the release profile of EC-RA microparticles is characterized by a sustained release where only around 20% of the antioxidant was liberated in the first 24 h. It would be recommended to perform the study for a longer period to determine the amount of rosmarinic acid released. Considering the release of EC rosmarinic acid loaded microparticles in octanol, sustained release was verified in the first 5 hours, where around 60% of the antioxidant was liberated in the first 2 h. For both release mediums no burst release phenomenon was observed in the release profiles.

Ideally, to compare the release from microparticles prepared by spray drying and solvent evaporation methods the same polymer and the same release conditions (e.g. mass of microparticles, volume of dissolution medium) should be used. However, as previous explained it was not possible to use the same wall material. Regarding the release conditions, different masses of microparticles and volumes of release medium were tested for both methods. To normalize the release by the amount of microparticles used, Figure 25 presents the release profile of both CMC and EC rosmarinic acid particles, in water, divided by the amount of microparticles used in each experiment. CMC rosmarinic acid microparticles prepared by spray drying led to a faster release comparing to EC rosmarinic acid microparticles prepared by o/w solvent evaporation. These results can be justified by the solubility of CMC in water.

The encapsulation of rosmarinic acid by spray drying was also performed by Casanova et al. using chitosan and modified chitosan. The release profile of chitosan rosmarinic acid loaded particles presented a faster release in water and octanol than the CMC and EC rosmarinic acid particles prepared in this work. Regarding modified chitosan as wall material, results revealed to be more satisfactory than chitosan but, in any case, CMC and EC had provided slower releases of rosmarinic acid. [170]. Microparticles of chitosan and CMC were prepared by Zhang et al. using bovine serum albumin as core material and emulsion phase separation method. Results evidenced that the combination of chitosan with CMC slows the release of the protein due to the interaction of both polymers. Additionally, Feng et all combined the use of EC with chitosan to entrap genipin, using spray drying, for anti-tuberculosis delivery. The release tests were performed in PBS (pH 7.4) and presented a slower release than the ones obtained in this work [174]. Therefore, the combination of chitosan with both wall materials could lead to slower release profile which may be suitable for cosmetic application. Kök et al. described the incorporation of a pesticide, aldicarb, in CMC microparticles. The release profile in water is very different from the one obtained in this work since it shows a slower release behavior. However, a different method, core material and release conditions were used which can justify the differences [175].

The incorporation of ascorbic acid in EC microparticles was performed by w/o solvent evaporation and the controlled release test was made in water. Results revealed a faster release than the particles prepared in this work using o/w solvent evaporation [176]. Theophylline was incorporated in hydrophilic matrix containing, among other substances, EC and CMC. The release test was performed in simulated intestinal fluid (pH 7.5) and results evidence that the presence of EC may retard the release of the drug from the matrix when low molecular weight CMC was used [177]. The controlled release results can also be compared with some of the studies described in chapter II using different types of delivery systems. Niosomes were used to evaluate the release of rosmarinic acid in PBS (pH 5.5). The *in vitro* release of RA from the niosomes and a niosome gel presented a slower release profile than the CMC and EC microparticles [139].

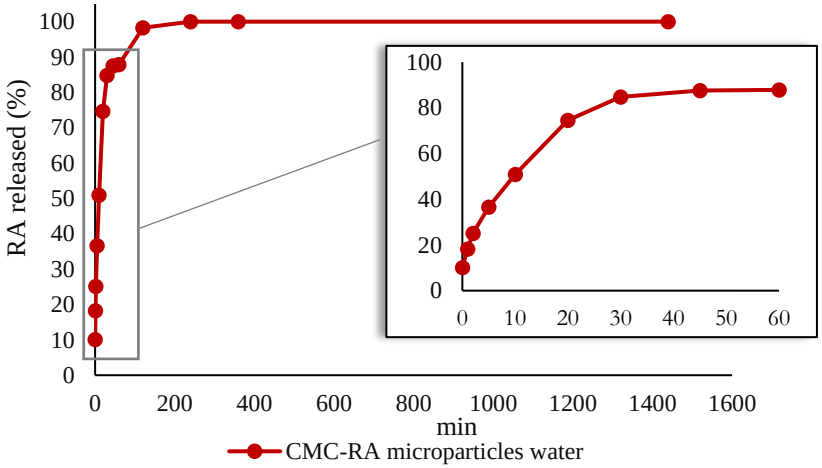


Figure 22 - Release profile of CMC-RA microparticles, prepared by spray drying, in water

Figure 23 - CMC-RA microparticles after 48h of release in octanol

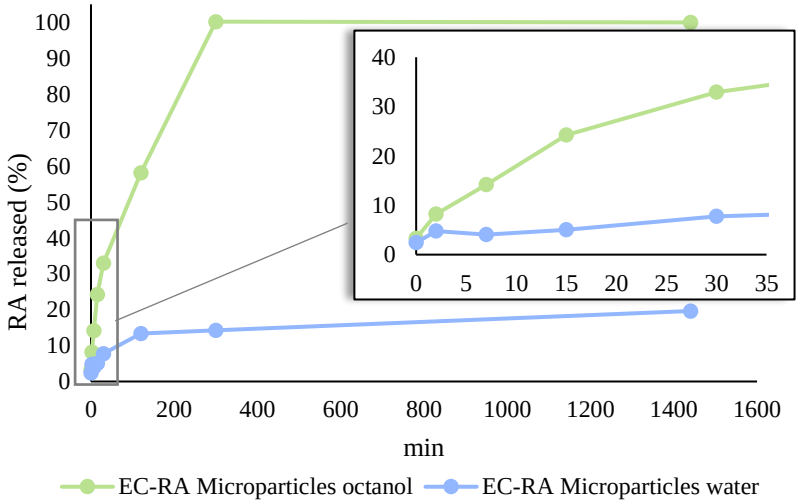


Figure 24 - Release profile of EC-RA microparticles, prepared by O/W solvent evaporation in water and octanol

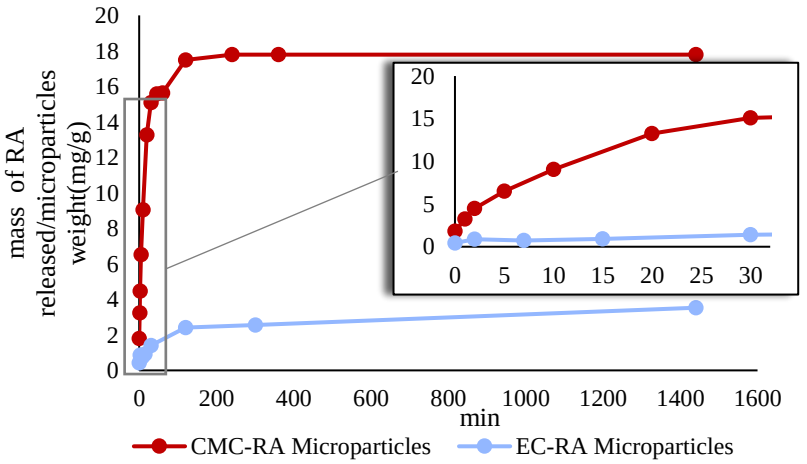


Figure 25 - Release profile of CMC-RA and EC-RA microparticles, prepared by spray drying and O/W solvent evaporation, respectively, in water

Rosmarinic acid was also encapsulated in poly(rutin) nanoparticles using PBS (pH 5.4) at 37 ºC as release medium. The release lasted for days while in the CMC microparticles prepared in this work it was completed in the first hours [138]. Liposomes with genistein were also tested in phosphate buffer (pH 7.4) at 37 ºC. Comparing both results, CMC-RA and EC-RA (in octanol) microparticles presented a faster release than liposomes however, when comparing to EC-RA microparticles placed in water the release of the antioxidant was slower [155]. SLN microparticles were used to entrap resveratrol and the release profile in water was very similar to the release profile of EC-RA microparticles in octanol for the first 3 hours [156]. The release of NLC carrying rutin from cream formulations was assessed in phosphate buffer (pH 5.5). Comparing to EC-RA and CMC-RA release in water, nanocreams revealed a slower release profile [158].

These preliminary studies of control released of rosmarinic acid using CMC and EC as wall material suggest that they can be used since both retard the release of the antioxidant. However, for cosmetic application usually a slow release profile is required. EC-RA microparticles placed in water presented the most interesting behavior because they only released around 20 % of their drug content in the first 24h. Therefore, if they remain stable in a cosmetic formulation without any more release of the antioxidant, the release of the residual rosmarinic acid could be triggered by mechanical forces when the product is applied.

V.4. Antioxidant activity

The antioxidant activity can be reduced by the encapsulation process or interactions with the wall material. Table 11 presents antioxidant activity of free rosmarinic acid and after being encapsulated. To perform evaluation, the ABTS radical scavenging assay was used and the amount of the remaining ABTS radical after reaction with the sample was measured by UV-Vis-spectrophotometry. The results are expressed in trolox equivalents (TE). The standard curve is presented in appendix 4.

Table 11 -Antioxidant activity of free rosmarinic acid and CC-RA microparticles

	Antioxidant activity (µM TE)
Free rosmarinic acid	208
CMC- RA microparticles	241
Free rosmarinic acid	143
EC- RA microparticles	197

According to Table 11 the free rosmarinic acid presented lower antioxidant activity than the encapsulated one. CMC and EC empty microparticles were also tested and it was observed that their presence interfered with the measurements although no antioxidant activity has been found in literature for both polymers. Therefore, the difference between antioxidant activity values of free and encapsulated rosmarinic acid should be lower. The same results were obtained by Carvalho et al regarding the sun protection factor of green coffee oil when encapsulated in corn syrup [146]. On the other hand, to prepare the free rosmarinic acid

solution with the same amount of rosmarinic acid as the microparticles sample, it was considered that the powder had the theoretical amount of antioxidant. This theoretical value was calculated taking into account that the ratio of rosmarinic acid and the polymer was constant for CMC particles and considering the amount of rosmarinic acid liberated after 24 hours for EC microparticles. However, the real mass of rosmarinic acid is most of the times different than theoretical value which may make the results of free and encapsulated antioxidant activity not so comparable. Despite all of this, it is well known that microencapsulation protects the antioxidant from degradation which enhances its bioavailability. In addition, several authors claim that after microencapsulation the core material still have a proper antioxidant activity [146–148].

VI. Conclusion

In this work, carboxymethyl cellulose and ethyl cellulose microparticles containing rosmarinic acid were prepared by spray drying and o/w solvent evaporation, respectively. Both types of microparticles were characterized regarding their size and shape. Encapsulation efficiency, product yield and drug loading were also calculated. The controlled release studies were performed during 24 h in water and octanol to simulate cosmetic vehicles. Antioxidant activity of the encapsulated rosmarinic acid was evaluated using ABTS radical scavenging assay.

UV-Vis spectrophotometry analytical method was used to quantify the amount of rosmarinic acid released from microparticles. All the measurements were performed at 324 nm. The standard curve equation in water was Abs = 0.059 ± 0.003 [RA] + 0.02 ± 0.03 and in octanol was Abs = 0.0457 ± 0.0009 [RA] - 0.002 ± 0.008. The limit of quantification in water and octanol was 1.66 and 0.700 mg/L of rosmarinic acid, respectively while, the limit of detection in water and octanol was 0.497 and 0.210 mg/L of rosmarinic acid, respectively. The analytical method was validated for both mediums. The intermediate precision, repeatability and accuracy studies showed no significant variations in the measurements and demonstrated that the analytical method reproduced reliable results.

Regarding particles size, considering the number distribution, CMC and CMC-RA particles had a mean size of 0.156 and 0.358 µm, respectively while, taking into account the volume distribution, CMC and CMC-RA microparticles had mean size of 13.49 and 10.90 µm, respectively. These results suggest a size heterogeneity of the powder or agglomeration. CMC-RA microparticles prepared by spray drying presented a smooth surface and a spherical regular shape. EC-RA microparticles prepared by o/w solvent evaporation presented an irregular shape as well as some roughness on the surface.

O/W solvent evaporation method presented higher product yield (95.4%) but low encapsulation efficiency (6.87%). On the other hand, spray drying microparticles showed higher encapsulation efficiency (90.8%) but lower product yield (38.4%)

The controlled release studies in water revealed that CMC-RA microparticles had a faster release (17.5 mg RA/g microparticles at 2 h) than EC-RA microparticles (2.41 mg RA/g microparticles at 2 h). In addition, EC-RA microparticles only released around 20% of their drug content in water after 24 h. The release of CMC-RA microparticles in octanol was not possible to assess but the release of EC-RA microparticles in this medium (58% at 2 h) was faster than in water (13%).

Finally, the antioxidant activity of encapsulated rosmarinic acid in CMC (208 µM TE) and EC (197 µM TE) is slightly higher than free rosmarinic acid solutions probably due to interference of the wall material in the measurements.

These preliminary studies of controlled release of rosmarinic acid using CMC and EC as wall materials suggest that they can be used for its microencapsulation. Both materials retarded the release of the antioxidant and they did not reduce its antioxidant capacity. However, for cosmetic application usually slow release

profiles are required. EC-RA microparticles placed in water presented the most interesting behavior because they only released around 20 % of their drug content in the first 24 h.

Although the satisfactory results obtained in this work, some other critical aspects should be considered and evaluated. To develop a delivery system not only the controlled release profile is important. The scale up and optimization of the encapsulation method is essential to obtain great amounts of powder as well as to assure its homogeneity, quality and reasonable price. The method by which the delivery system is incorporated in a cosmetic formulation must also be assed to assure the stability of the microparticles and the remaining formulation. In addition, the right concentration of microparticles in the final formulation must be calculated combining the drug loading capacity of the particles with proper amount of active ingredient in the formulation. Rheological properties, appearance and texture of the final formulation must also be taking into account. Microparticles must also be resistant to process conditions (e.g. stirring) however, they may have to be able to crush when the product is applied on the skin. Storage and transport conditions of the product may also be considered to guarantee the stability of the delivery systems at different temperatures, pressures and the presence of light. Health safety of the microparticles and their materials should be guaranteed as well. Finally, the consumer's perspective and the flexibility of the delivery system to incorporate different core materials are important factors to consider due to the rapid change of market trends. Therefore, this study only consisted in a small part of a long, complex and multidisciplinary process that has to combine several variables that can affect the success of the micro particulate product.

Future work and limitations

Some of the main limitations found in the presented work were related to the availability of the equipment and time management. Since three different persons were using the same magnetic stirrer and spectrophotometer, it was necessary to conciliate everyone in order to get the best possible results. Another limitation was the handling of coconut oil. Initially it was planned to use coconut oil as release medium for microparticles in order to better simulate cosmetic vehicles. However, this reagent was solid at room temperature and it only turned into a transparent liquid when melted at around 40 ºC. When the temperature was lower, the liquid became translucent ending up in the solid state. For this reason, it was impossible to perform measurements in the spectrophotometer. The poor dissolution of CMC in octanol and consequently the no release of rosmarinic acid from the microparticles in the first 24 h was another contretemps. The low amount of powder obtained by o/w solvent evaporation method and difficulties of scaling up the process also limited this work. Finally, the impossibility of determining size distribution of EC-RA microparticles is another gap in this study.

These preliminary studies of controlled releas of rosmarinic acid using CMC and EC as wall materials suggest that they can be used for its microencapsulation. Both materials retard the release of the antioxidant and they did not interfere with its antioxidant capacity. Spray drying is probably the best encapsulation method when an industrial application for the microparticles is intended since the scale up may be easier. However, it is suggested an optimization of the operational conditions. Additionally, different wall materials and cross linking agents should be tested always considering their safety for cosmetic application. Regarding the o/w solvent evaporation method, an optimization of the method and its scale up is required to obtain a larger amount of microparticles and better encapsulation efficiencies. Some of the steps that should be optimized are the separation of the microparticles from polyvinyl alcohol solution by means of filtration or centrifugation, the concentrations of antioxidant and the polymer, concentration of the emulsifier agent, agitation rate, temperature, time for solvent evaporation.

For cosmetic application, slower release profiles are required. After obtaining satisfactory release profiles in simple release mediums simulating cosmetic vehicles, microparticles should be incorporated in basic cosmetic formulation to test their long-term stability and the release profile of the antioxidant. At this point, the extraction of the antioxidant from natural sources could be studied and optimized. The extract should be encapsulated and subjected to the same tests as the pure rosmarinic acid. Both results should be compared.

The antimicrobial activity of rosmarinic acid microparticles could also be assessed against some microorganisms reported in literature.

Skin permeation and distribution of rosmarinic acid within skin layers should also be assessed using Franz diffusion cells as well as, safety and irritancy of the formulation. Finally, the good performance of the product should be tested *in vivo* to assure the success of the product.

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Appendix 1

Table A - Example of cosmetic products with delivery systems and antioxidants [195], [196]

Trade name	**Active ingredient** ***delivery system***	**Proposed use**	**Name of the company**
Revitalift	Pro-Retinol A *Nanosome*	Anti-wrinkle	L'Oreal
Bioperformance Crème Super Régénérante Absolue	Gamma linolenic acid *Nanocapsules*	–	Lancôme
Rénergie Microlift	Micro filters (silica and protein) *Nanoparticles*	Antiaging Moisturizer	L'Oreal
Advanced Night Repair Protective Recovery Complex	*Liposome*	Skin repair	Estée Lauder
Rovisome ACE Plus	Ascorbyl palmitate, tocopherol, retinol *Liposome*	Anti-aging, wrinkle reduction	Rovi Cosmetics International GmbH
Lancôme Soleil Soft-Touch Anti-Wrinkle Sun Cream SPF 15	Vitamin E, panthenol *Nanocapsules*	Revitalizing, anti-aging	L'Oreal
Lipobelle Soyaglycone	Genstein *Liposome*	Antioxidant	Mibelle Biochemistry
Cutanova Nano Repair Q10 Cream	Coenzyme Q10 *Nanostructured lipid carriers*	Revitalizing, anti-aging	Dr. Rimpler GmbH
Collagen Stimulator Factor MAP®	Vitamin C *Nanocapsules*	Stimulation of collagen production	Cosmetochem

Appendix 2

Table B - Examples of delivery systems with pure antioxidants with cosmetic relevance

Class of active ingredient	Active ingredient	Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Apigenin	1. Lipoid S 75, ethanol and propylene glycol 2. Lipoid S 75 3. Lipoid S 75 and Tween-80	1. n.s. 2. Conventional mechanical dispersion 3. Conventional mechanical dispersion	1. Ethosome 2. Liposome 3. Transfersome	- *Entrapment efficiency*: 61.69% to 85.21% for ethosomes, 89.55% for liposomes and 81.93% for transferosomes. - Ethosomes showed superior skin targeting both in vitro and in vivo. - Ethosomes produced the strongest effect on UVB-induced skin inflammation.	[178]
		PLGA[1]	Solvent displacement	Nanoparticle *(101.3 nm)*	- *Entrapment efficiency*: 87.2%. - Initial burst release up to 16 h followed by controlled release for up 72 h. - Loaded nanoparticles produced better effects than free apigenin.	[179]
	- Berberine - Gallic acid	Agar/gelatin	n.s.	Microcapsule *(17 µm and 22 µm)*	- *Entrapment efficiency*: 78.16% for berberine and 70.28% for gallic acid. - In vitro drug delivery showed the delivery of berberine from microcapsule treated textiles into nude mice skin.	[180]
Anticellulite	Caffeine	Phosphatidylcholine and cholesterol	Thin film hydration	Liposome	- *Drug loading*: 37% to 58%. - Higher skin permeation of liposomal cabopol gel formulation. - Liposomes stored at 2 to 8 °C were more stable.	[131]
Antioxidant	Catechins	Phosphatidylcholine, cholesterol (CH), deoxycholic acid (DA) and/or dicetyl phosphate and/or PEG, ethanol,	n.s.	Liposome *(121 – 216 nm)*	- *Entrapment efficiency*: 39% for CH-liposomes, 65% for DA-liposomes, 93% for ECGG[2] loaded DA,CH-liposomes. - Intercellular spaces within the SC[3] are the major pathways for catechin delivery from liposomes. - ECGG showed the highest encapsulation rate and in vivo skin deposition level in liposomes.	[181]

n.s.- not specified; [1] PLGA- poly(lactic-co-glycolic acid); [2]ECGG- (−)-Epigallocatechin-3-gallate; [3] SC- stratum corneum

Table B - Examples of delivery systems with pure antioxidants with cosmetic

Class of active ingredient	Active ingredient	Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Ellagic acid (EA)	1. Sorbitan monostearate, polyethylene glycol 2. sorbitan monosterarate, cholesterol	Reverse phase evaporation	Niosome *(124–752 nm)*	- *Entrapment efficiency:* 1.35% to 26.75%. - The penetration of EA from the niosomes depended on vesicle size, the amount of EA entrapped and the added solubilizers.	[160]
Antioxidant pure and extracted	Gallic acid	*Elastic:* Tween 61, cholesterol, ethanol *Non-elastic:* Tween 61, cholesterol, PBS	Modified chloroform film	Niosome *(200–400 nm)*	- *Entrapment efficiency*: 55% and 24 % for pure gallic acid and gallic acid in the semi purified fraction in elastic niosomes; 30% and 20% for pure gallic acid and gallic acid in the semi purified fraction in non- elastic niosomes. - Elastic niosomes exhibited higher amounts of gallic acid through rat skin.	[182]
Antioxidant	Genistein	1. soya phosphatidylcholine 2. soya phosphatidylcholine and sodium deoxycholate	Rotary evaporation and extrusion method	1. Liposome 2. Transfersome	- *Entrapment efficiency:* 80% for liposome and for transfersomes. - Percutaneous delivery of elastic liposomes was influenced by existence of hair follicles. - The greater permeation rate and deposition values of genistein were observed from the elastic liposomes than conventional liposomes in haired skin.	[155]
	Lycopene	Eumulgin® SG, orange wax and rice bran oil	High pressure homogenization	NLC	- *Entrapment efficiency*: 100%. - Biphasic release profile: relatively fast release during the first 6 h followed by a sustained release during the next 18 h. - Increased stability of lycopene and slow degradation rate when stored at low temperatures - The occlusive properties of NLC increased with increasing lycopene loading.	[157]
- Whitening agent -Antioxidant	Niacinamide (NA)	Hydrogenated lecithin, cholesterol, ceramide 3 and dipotassium glycyrrhizate	High-pressure homogenization	Liposome Bounsphere™ *(200 nm)*	- Higher skin permeability of transferosomes than conventional liposomes or the phosphate buffer solution - 2% NA-loaded transferosomes enhanced skin whitening	[162]

Table B - Examples of delivery systems with pure antioxidants with cosmetic

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Phyllanthin	Chitosan	Simple coacervation	Microcapsule *(5.32 µm)*	- *Entrapment efficiency:* 60%. - The release of phyllanthin from the microcapsules was found to be more than 60% after 120 h. - Microcapsules showed a stronger anti-oxidation potential on both human fibroblasts and keratinocytes.	[153]
	Quercetin Rutin	Phosphatidylcholine, ceramide-3, cholesterol and oleic acid	Thin-film hydration	Liposome *(144 nm)*	- *Entrapment efficiency:* 33%- 57% for rutin, 25% - 40% for quercetin, approximately. - Rutin had better in vitro release properties while quercetin demonstrated greater skin permeability. - Liposome-in-hydrogel complex systems improved skin permeability.	[183]
	Quercetin (QT)	Soya lecithin, glyceryl monostearate, stearic acid and media chain triglyceride	Emulsion evaporation (solidification at low temperature)	NLC *(215.2 nm)*	- *Entrapment efficiency:* 90% - *Drug loading:* 3.05% - QT-NLCs could promote the permeation of QT, increase the amount of QT retention in epidermis and dermis, and enhance the effect of anti-oxidation and anti-inflammation exerted by QT.	[184]
		PLGA[1] and tocopheryl polyethylene glycol (TPGS)	Nanoprecipitation	Nanoparticle *(100-150 nm)*	- *Entrapment efficiency:* 82%. - *Drug loading:* 8.62%. - Quercetin NPs[4] can significantly block UVB irradiation - PLGA-TPGS NPs can get through epidermis and reach dermis.	[185]
		Lectinin and chitosan	n.s.	Nanoparticles *(95.3 nm)*	- *Entrapment efficiency*: 49%. - *Drug loading*: 2.45%. - Quercetin NPs showed higher permeation ability and increased accumulation of quercetin in the skin, especially in the epidermis.	[186]

n.s.- not specified; [1] PLGA- poly(lactic-co-glycolic acid); [4] NP- Nanoparticles;

Table B - Examples of delivery systems with pure antioxidants with cosmetic relevance

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Quercetin	Tristearin and phosphatidylcholine	n.s.	Lipid microparticle *(10 - 45µm)*	- *Drug loading:* 12.1%. - The quercetin release from the microparticle did not exhibit burst-effect phenomena.	[159]
	Resveratrol and curcumin	PCL[5], sorbitan monostearate, grape seed oil	n.s.	Lipid core nanocapsules *(184 nm)*	- *Entrapment efficiency*: close to 100% - Resveratrol penetrated into deeper skin layers when the co-formulation was applied - Smaller amounts of curcumin were released from nanocapsules compared to resveratrol	[140]
	Resveratrol Curcumin α-Tocopherol	Tween 60	n.s.	Niosomes *(471 - 565 nm)*	- The antioxidants in vitro percutaneous permeations appeared to be controlled and improved respect to the corresponding free solutions used as control. - The antioxidants combinations resulted in a promoted ability to reduce free radicals.	[33]
	Resveratrol	Chitosan Tristearin and hydrogenated phosphatidylcholine	Melt emulsification and sonication	Lipid Microparticles *(5.7 µm)*	- *Drug loading:* 4.1%. - Chitosan coating changed the LM [6] surface charge. - Significant enhancement in the in vivo permeation of resveratrol.	[136]
		Phosphatidylcholine and chitosan	n.s.	Lipid Nanoparticle *(280 - 558 nm)*	- *Entrapment efficiency:* 83% (highest). - The chitosan coating was seen to increase the stability of the liposomes by preventing their aggregation. -A increased skin-permeation efficiency with the coating was observed.	[137]
		1. Soy phosphotidycholine 2. Cholesterol, glycerol monooleate and/or polyglyceryl-3 dioleate	n.s.	1. Liposome 2. Niosome *(200 nm)*	- *Entrapment efficiency*: 79% to 84%. - Niosomes formulated with Plurol oleique or Peceol showed a better behavior than liposomes in the cutaneous delivery.	[141]

n.s.- not specified; [5] PCL- poly(ε-caprolactone); [6] LM- Lipid Microparticle;

Table B - Examples of delivery systems with pure antioxidants with cosmetic

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Resveratrol	Cetylpalmitate and tricaprin	Hot melt homogenization	SLN *(379- 472 nm)*	- *Entrapment efficiency*: 51% to 53% - The presence of tetradecyl-c-cyclodextrin in SLN formulation improved nanoparticle characteristics.	[142]
		Phosphatidyl-choline cholesterol	n.s.	Liposome *(115 and 305 nm)*	- Resveratrol is more deeply inserted in cationic liposomes than in zwitterionic liposomes. - Cell viability was not affected by the administration of liposomial resveratrol.	[143]
		1. soy phosphatidylcholine 2. PCL 3. Lipid glyceryl dibehenate, coconut fat, coconut oil, medium-chain triglycerides	1. Lipid film hydration 2. Precipitation 3. High-pressure homogenization	1. liposomes *(189.4 nm)* 2. polymeric lipid-core nanocapsules *(266 nm)* and nanospheres *(207nm)* 3. NLC *(176.0 nm)*	- *Entrapment efficiency:* >98%. - In the dark, the skin penetration profiles were very similar, but under UVA radiation the Resveratrol-loaded nanocarriers showed increasing amounts in the total epidermis.	[144]
		1. Glyceryl behenate 2. Glyceryl behenate and Miglyol oil	High shear homogenization	1. SLN *(287.2 nm)* 2. *NLC (110.5 nm)*	- *Entrapment efficiency:* 72% to 81% - NLC are more efficient in carrying resveratrol to the epidermis. - Resveratrol-NLC produced less ROS in cytofluorometric.	[145]
		Glyceryl behenate, hydrogenated soya bean lecithin	Melt-emulsification	SLN *(180 nm)*	- The release profile of resveratrol showed a biphasic pattern. - Size below 180 nm move through the cell membrane, distribute throughout the cytosol, move successively among different cellular levels and localize in the perinuclear region without inducing cytotoxicity.	[156]
	Rosmarinic acid	Poly rutin	Microemulsion polymerization/ crosslinking	Nanoparticle *(631 nm without RA)*	- Showed release capability for up to 6 days by releasing 85% of the loaded RA. - Highly negatively charged p(RT)[7] particles.	[138]
		Sorbitan and cholesterol	Emulsion solvent evaporation	Niosome (814.2 nm)	- *Entrapment efficiency:* 50% to 65% - After 24 h, the release was 49.81±1.76%. for niosomal gel.	[139]

n.s.- not specified; [7]p(RT)- Poly rutin

Table B - Examples of delivery systems with pure antioxidants with cosmetic

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Rosmarinic acid	PCL[5]	Emulsion solvent evaporation	Microspheres *(~ 7-15 µm)*	- *Entrapment efficiency*: 22 to78%. - Emulsions containing RA-loaded PCL microspheres showed a better long-term stability of the RA compared with those containing only RA.	[56]
	Rutin (RT)	White soft paraffin and Liquid paraffin, pifil® GC, or Gelucire®50/13, or Plurol® stearique WL 1009, or Tefose® 2000 CG	Probe sonication	NLC *(85 -319 nm)*	- *Entrapment efficiency*: 90% to 97% - Progressive increase in occlusive properties of the tested formulations from 6–48 h. - An initially rapid release during the first 6 h (burst effect). - Non-significant effect of RT concentration on sun-blocking.	[158]
		Gelatin	Two-step desolvation	Nanoparticle *(398 nm)*	- *Entrapment efficiency:* 52% - Increased antioxidant activity by 74% relative to free-rutin solution. - Rutin upgraded the Sun Protection Factor by 48%.	[134]
	Vitamin C	Soybean phosphatidylcholine, cholesterol, tween-80 and pectin	Film evaporation combined with dynamic high pressure microfluidization	Liposome coated with pectin *(~123.5 nm)*	- *Entrapment efficiency*: 48% to 50%. - Low aggregation, oxidation of lipid and leakage of vitamin C from liposomes. - Improvement of skin permeation.	[187]
		PLGA[1]	n.s.	Nanosphere *(110–170 nm)*	- *Entrapment efficiency*: > 90% - In the first 24 days of the degradation, less than 10% of the encapsulated ascorbic acid have been released. - The overall quantities of the encapsulated ascorbic acid have been released in 8 weeks of the degradation.	[188]
		Chitosan	Interfacial/emulsion reaction	Microcapsule *(2–5 µm)*	- The release rate of vitamin C was dominated by the core content of vitamin C and the crosslinking density.	[135]

n.s.- not specified; [1]PLGA- poly(lactic-co-glycolic acid); [5] PCL- poly(ε-caprolactone);

Table B - Examples of delivery systems with pure antioxidants with cosmetic

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Antioxidant	Vitamin E	Chitosan	Spray-drying	Microspheres	- *Entrapment efficiency*: 78% - Microspheres showed burst release at the 5th minute after application, however release lasted for 6 h. - Decreased effect of skin roughness and increases in skin moisture and elasticity.	[148]
		PLGA[1] PCL[4]	Nanoprecipitation	Nanocapsule *(165 and 172 nm)*	- *Entrapment efficiency*: 98% and 97%. - Scale up produced a slight increase in size and no drug encapsulation efficiency reduction.	[189]
		Poly(l-lactic acid)	Solvent evaporation	Microcapsule	- Vitamin E was well enveloped with lower amount of high molecular weight PLLA[7], PLLA: vitamin E at 3:1.	[149]

[1] PLGA- poly(lactic-co-glycolic acid); [7]PLLA- Poly(l-lactic acid); [4] PCL- poly(ε-caprolactone)

Table C - Examples of delivery systems with cosmetic ingredients

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Extract	*C. asiatica*	Gelatin	n.s.	Nanoparticle *(115.0 nm)*	- *Entrapment efficiency*: approximately 67%. - High flux through mouse skin and also remained at relatively large concentrations in the derma. - Lower cytotoxicity in human skin fibroblast cells.	[133]
	Cortex Moutan (Gallic acid)	Chitosan/sodium alginate	Emulsion-chemical cross-linking	Microcapsule *(1-10 µm)*	- *Entrapment efficiency*: 80%. - The gallic acid release reached approximately 75% and 55% during the initial phase of first 24 h and achieved a constant level of 98% and 85% for pH 5.0 and pH 5.4 PBS, respectively.	[152]
	Cortex Moutan	Chitosan	Emulsion-chemical cross-linking	Microcapsule *(8.9 µm)*	- *Maximum yield:* 87% - The optimum condition for microcapsule preparation was core–shell ratio = 1:2; chitosan concentration = 6% (w/v); agitation speed = 1100 rpm; and cross-linking time = 90 min.	[151]
	Curcuma longa	1. soya phosphatidylcholine and cholesterol 2. soya phosphatidylcholine and ethanol 3. soya lecithin and sodium deoxycholate	1. Film hydration 2. Cold method 3. modified lipid film hydration (rotary evaporation)	1. Liposome *(213 - 262 nm)* 2. Ethosome *(167- 195 nm)* 3. Transfersome *(176 - 199 nm)*	- *Entrapment efficiency:* 45% for liposomes, 70% for ethosomes and 82% for transferosomes - The cream efficacy was in the order transfersomal > ethosomal > liposomal > free *C. longa* > empty transfersome > empty ethosome > empty liposome > base cream.	[165]
	Fraxinus angustifolia	1. soybean phospholipids, ethanol 2. soybean phospholipids and Transcutol or ethylene glycol	n.s.	1. Ethosomes 2. Phospholipid vesicles *(100-200 nm)*	- *Entrapment efficiency*: 39% for ethosomes, 37% and 40% for lipid vesicle with transcutol and ethylene glycol, respectively. - Ethylene glycol lipid vesicles are a promising carrier for natural extract to be used in the pharmaceutical and cosmetic.	[161]

n.s.- not specified

Table C - Examples of delivery systems with cosmetic ingredients

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Extract	*Garcinia mangostana* (GML)	Ethylcellulose blended methylcellulose	Solvent displacement	n.s.	- *Entrapment efficiency*: 99%. - *Drug loading:* 49.73%. - Encapsulated and free GML in the cream base penetrated deeper into hair follicles but encapsulated distributed more homogeneously on the stratum corneum.	[164]
	Green coffee oil	Corn syrup, Octenyl succinic anhydride modified starches	Electrostatic layer-by-layer deposition Spray drying	Microcapsule *(15-20 µm)*	- *Entrapment efficiency*: > 86%. - The sun protection factor of micro particles ranged from 1.52 to 2.45, close to the pure green coffee oil.	[146]
		Arabic gum	Spray drying	Microcapsule *(4 - 11 µm)*	- Drug loading: 10% and 30%. - Microcapsules showed higher antioxidant activity when compared to pure green coffee oil.	[147]
	Helichrysum stoechas (L.) Moench	Polycaprolactone diol	Double emulsion/evaporation Lyphiolization	Microcapsule *(30-100 µm)*	- *H. stoechas* hydroalcoholic extract demonstrated antioxidant potential and the viability of its microencapsulation justifies its possible application in the cosmetic industry.	[150]
	L. stoechas ssp. uisieri *L. pedunculata*	PLGA[1]	Modified-spontaneous emulsification solvent diffusion	Nanoparticles *(302 and 304 nm)*	- *Entrapment efficiency:* > 96% - The epidermal permeation of both extracts through human epidermis and their in vitro cytotoxicity in human keratinocytes were suggestive of low risk.	[190]
	Polygonumaviculare L.	Dioleoylphosphatidylcholine, cholesterol, MPB-PE[9] and peptide	Thin-film hydration	Liposome *(150 nm)*	- *Entrapment efficiency*: 83%. - Peptide- liposomes improved cellular uptake compared with the typical liposomes. - Skin permeation of peptide-liposomes was higher. -Peptide-liposomes were more effective in depigmentation and anti-wrinkle studies.	[163]

n.s.- not specified; [1]PLGA-poly(lactic-co-glycolic acid); [8] 1,2-di-(9Z-octadecenoyl)- sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide];

Table C - Examples of delivery systems with cosmetic ingredients

Class of active ingredient	Active ingredient	Delivery Material	Preparation method	Delivery system *(size)*	Main Results	Ref
Extract	Rice bran oil	Sorbitan monostearate and PCL[5]	n.s.	Lipid-core Nanocapsule *(200 nm)*	- This formulation was able to prevent ear edema induced by UVB irradiation by 60 ± 9%.	[102]
Essential oil	*Nigella sativa L.*	Hydrogenated palm oil, Softisan 154	High-pressure homogenization after starring and ultra-trax homognization	SLN	- High physical stability at various storage temperatures during 3 months of storage. - SLN formulations are suitable carriers in cosmetic fields and pharmaceutical.	[191]
Enzyme	Papain	1. Tween 61, cholesterol and sodium cholate 2. PLGA[1]	1. Thin film hydration 2. Water–oil–water emulsion solvent evaporation	1. Niosome 2. Nanosphere *(221–520 nm)*	- Papain loaded elastic niosomes in gel formulation exhibited accumulated amounts and fluxes higher than non-elastic niosomes. - No irritation on rabbit skin.	[132]
Essential oil	Thyme oil	PLA[10]	Coacervation	Microcapsule *(36 µm)*	- *Entrapment efficiency:* 65%. - The release of thymol is faster in the first hour and remains almost constant in the next days.	[154]

n.s.-not specified; [1]PLGA-poly(lactic-co-glycolic acid); [5] PCL- poly(ε-caprolactone); [10] Polylactic acid

Appendix 3

Analytical method validation

A) Water Standard curve

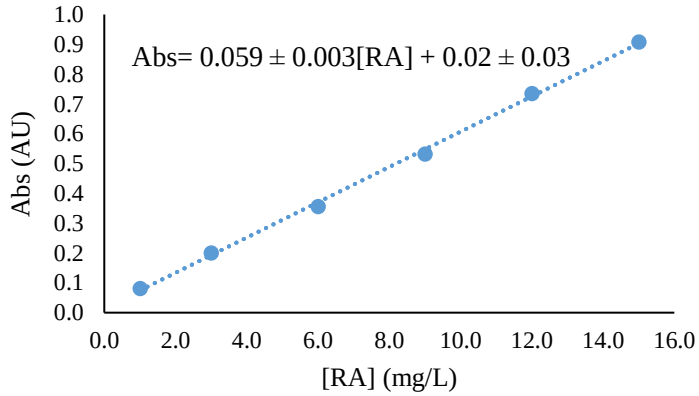

Figure A - Standard curve for quantification of rosmarinic acid in water by UV-Vis- spectrophotometry

Table D - Intermediate precision (%CV) of the UV-Vis-spectrophotometry method for RA quantification in water at three concentration levels

	Day 1		Day 2		Day 3				
[RA] (mg/L)	**Assay 1 (AU)**	**Assay 2 (AU)**	**Assay 1 (AU)**	**Assay 2 (AU)**	**Assay 1 (AU)**	**Assay 2 (AU)**	**Mean (AU)**	**SD (AU)**	**Intermediate Precision (%)**
3.0	0.2006	0.2006	0.175	0.1751	0.1923	0.1924	0.19	0.01	5.6
9.0	0.5011	0.5011	0.5161	0.5161	0.5318	0.5327	0.52	0.01	2.5
15.0	0.9087	0.9066	0.8697	0.8687	0.8874	0.8874	0.89	0.02	1.8

Table E- Accuracy (%R) of the UV-Vis-spectrophotometry method for RA quantification in water at three concentrations levels

[RA] expected (mg/L)	**Assay 1 (AU)**	**Assay 2 (AU)**	**Assay 3 (AU)**	**Assay 4 (AU)**	**Assay 5 (AU)**	**Assay 6 (AU)**	**Mean (AU)**	**SD (AU)**	**[RA] obtained (mg/L)**	**Accuracy (%)**
3.0	0.1937	0.1914	0.1923	0.1915	0.1924	0.1915	0.1921	0.0008	2.987	99.58
9.0	0.5166	0.5161	0.5160	0.5161	0.5167	0.5168	0.5164	0.0003	8.469	94.10
15.0	0.8891	0.8874	0.8847	0.8841	0.8874	0.8877	0.887	0.002	14.7	98.2

Table F - Repeatability (%CV) of the UV-Vis-spectrophotometry method for RA quantification in water at three concentration levels

[RA] (mg/L)	Assay 1 (AU)	Assay 2 (AU)	Assay 3 (AU)	Assay 4 (AU)	Assay 5 (AU)	Assay 6 (AU)	Mean (AU)	SD (AU)	Repeatability (%)
3.0	0.175	0.1738	0.1756	0.1737	0.1751	0.1738	0.1745	0.0008	0.4339
9.0	0.5016	0.5007	0.5011	0.5011	0.501	0.5017	0.5012	0.0003	0.06912
15.0	0.8697	0.8687	0.869	0.8674	0.8674	0.8671	0.868	0.001	0.112

B) Octanol standard curve

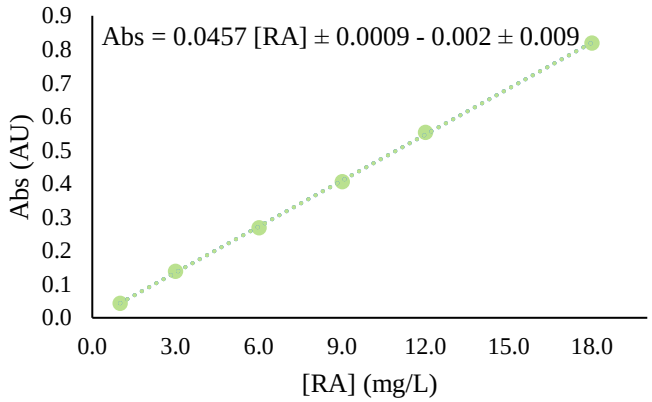

Figure B - Standard curve for quantification of rosmarinic acid in octanol by UV-Vis-spectrophotometry

Table G - Intermediate precision (%CV) of the UV-Vis-spectrophotometry method for RA quantification in octanol at three concentration levels

	Day 1		Day 2		Day 3				
[RA] (mg/L)	Assay 1 (AU)	Assay 2 (AU)	Assay 1 (AU)	Assay 2 (AU)	Assay 1 (AU)	Assay 2 (AU)	Mean (AU)	SD (AU)	Intermediate Precision (%)
3.0	0.1378	0.139	0.1561	0.1556	0.1315	0.1314	0.14	0.01	7.2
9.0	0.4045	0.4072	0.4129	0.4124	0.3945	0.3951	0.404	0.007	1.83
18.0	0.8184	0.8184	0.8377	0.8371	0.821	0.8202	0.825	0.008	1.03

Table H - Accuracy (%R) of the UV-Vis-spectrophotometry method for RA quantification in octanol at three concentrations levels.

[RA] expected (mg/L)	Assay 1 (AU)	Assay 2 (AU)	Assay 3 (AU)	Assay 4 (AU)	Assay 5 (AU)	Assay 6 (AU)	Mean (AU)	SD (AU)	[RA] obtained (mg/L)	Accuracy (%)
3.0	0.1295	0.1298	0.1315	0.1297	0.1314	0.1342	0.131	0.002	2.92	97.2
9.0	0.3945	0.3951	0.398	0.3985	0.3979	0.4004	0.397	0.002	8.75	97.2
18.0	0.8147	0.8142	0.817	0.8187	0.821	0.8202	0.818	0.003	17.9	99.7

Table I - Repeatability (%CV) of the UV-Vis-spectrophotometry method for RA quantification in octanol at three concentration levels

[RA] (mg/L)	Assay 1 (AU)	Assay 2 (AU)	Assay 3 (AU)	Assay 4 (AU)	Assay 5 (AU)	Assay 6 (AU)	Mean (AU)	SD (AU)	Repeatability (%)
3.0	0.1568	0.155	0.154	0.1561	0.1556	0.1534	0.155	0.001	0.755
9.0	0.4129	0.415	0.4136	0.4124	0.4143	0.4159	0.414	0.001	0.290
18.0	0.8377	0.8371	0.8383	0.841	0.8435	0.8425	0.840	0.002	0.292

Appendix 4

Antioxidant activity

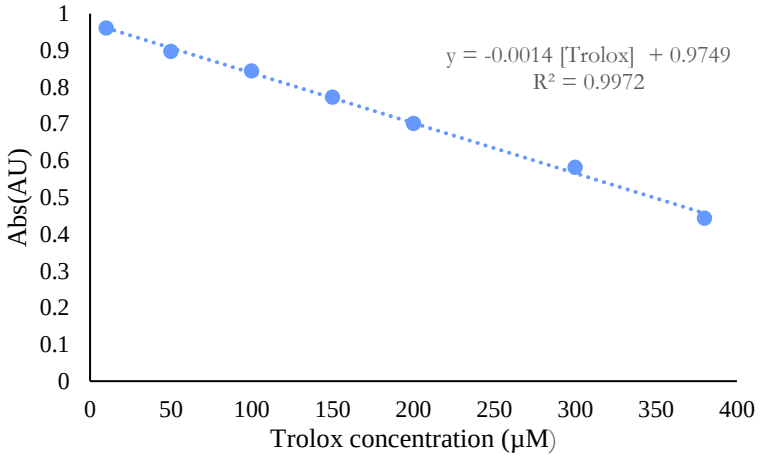

Figure C - Standard curve for quantification of ABTS radical by UV spectrophotometry

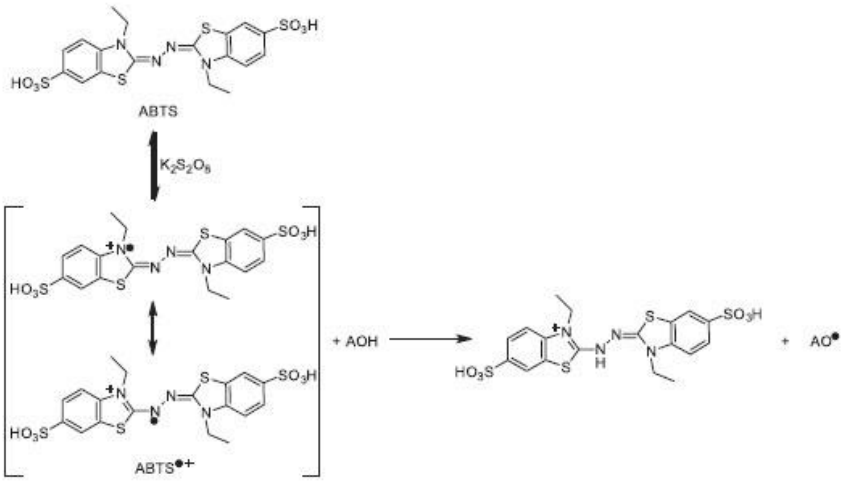

Figure D – ABTS oxidation by potassium persulfate and its possible reaction with an antioxidant molecule (AOH) [197]

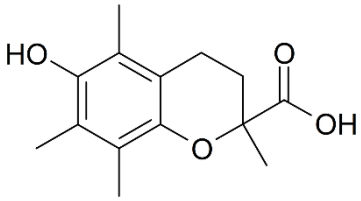

Figure E- Trolox chemical structure