Integrated Master in Bioengineering

Specialization in Biological Engineering

Microencapsulation of Eugenol - A promising compound for incorporation in versatile products

Dissertation for Master Degree

of

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Developed within the discipline of Dissertation

conducted at

Laboratory for Process Engineering, Environment, Biotechnology and Energy

lepabe
Laboratory for Process Engineering, Environment, Biotechnology and Energy

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July of 2017

U.PORTO
FEUP FACULDADE DE ENGENHARIA UNIVERSIDADE DO PORTO

Acknowledgments

I thank to Laboratory for Process Engineering, Environmental, Biotechnology and Energy (LEPABE) from Faculty of Engineering from University of Porto (FEUP) for providing the facilities, the equipment and the materials employed in this work as well as for the supervision of this project by Prof. Lúcia Santos. Moreover, I am also thankful to her for all the resources she provided, and for all the support, help, guidance and availability that she provided me during this project. I am grateful as well to all the research group members, professors and students from the Chemical Engineering Department (DEQ), for all their friendship and kindness. For the E201 laboratory group, I would like to thank for all the availability they provided in the laboratory. Further, I also wish to acknowledge Engineer Filipa Paulo for her advices and collaboration during the research and lab execution of this project.

Especially, I want to thank my parents for all the tireless encouragement, love, and patience they have been gave me for the last months. Additionally, I want to make a specially thank to my best friend, Rita Machado, who never left my aside and gave me the support and the strength until the end of the project. Lastly but no less important, I am also thankful to a few course friends, Ana Rodrigues, Catarina Pimentel and Isabel Oliveira, for sharing this academic phase with me and for the unconditional friendship.

This work was in part funded by projects (i) POCI-01-0145-FEDER-006939 (Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, UID/EQU/00511/2013) - funded by FEDER through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) – and by national funds through FCT - Fundação para a Ciência e a Tecnologia and (ii) NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION, funded by FEDER - Fundo Europeu de Desenvolvimento Regional, through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and Programa Operacional Regional do Norte (NORTE2020).

"You can throw your hands up
You can beat the clock
You can move a mountain
You can break rocks
You can be a master
Don't wait for luck
Dedicate yourself and you can find yourself
Standing in the hall of fame"

The Script

Abstract

The cosmetic and personal care products sector is an international market showing great economic expansion. The interest in added-value, natural and biological products in cosmetics and foods created the demand for new products as well as the reformulation of others with biological and functional ingredients. Furthermore, in the food sector, the demand of functional food products that contributes for a balanced diet includes the growing interest of consumers about the benefits that certain ingredients have. Moreover, in order to be effective in such industries, trying to avoid their competitiveness, the products that are commercialized need to be differentiate, which can be accomplished using emergent technologies such as microencapsulation. For instance, essential oils, due to their volatility and low stability since they are easily oxidized or decomposed in the presence of oxygen, light, enzymatic degradation, or heat, during processing, utilization and storage, are normally encapsulated to overcome these drawbacks. Therefore, there are numerous possibilities to use microencapsulation as a technique to obtain products with high added value, such as double emulsion solvent evaporation technique, which was the method used in the present study.

The purpose of this project is to prepare and characterize eugenol-loaded ethyl cellulose microparticles by double emulsion solvent evaporation technique; as well as to evaluate the influence of selected formulation parameters on the characteristics of the final microparticles. It was also accomplished the sustained release of eugenol from ethyl cellulose microparticles in four different carriers: ultra-pure water and octanol in order to simulate hydrophilic and lipophilic cosmetic vehicles, and acid and basic mediums to recreate the gastric and intestinal fluids. Specific aims included the validation of the analytical method, as well as to determine quantification and reliability parameters. The main results obtained for the analytical method were good linearity and good precision as well as lower limits of detection and of quantification, for all the four mediums investigated. Regarding the characterization parameters, the product yield ranged between 77.5 ± 6.1% and 82.4 ± 2.6%; the encapsulation efficiency between 76.4 ± 21.6% and 93.3 ± 0.2%; and the water content ranged between 48.1 ± 7.2% and 87.5 ± 0.6%. Additionally, the prepared microparticles showed sizes ranging between 128 ± 0.2 μm and 850 ± 19.1 μm for the overall formulations tested, and the microparticles were generically spherical, polydisperse, porous and superficially rough. Moreover, the highest final accumulated releases after 24 hours were 77% in ultra-pure water, 68% in the basic medium, 91% in the acid medium, and 50% in octanol.

Keywords: Microencapsulation, eugenol, ethyl cellulose, dichloromethane, methanol, polyvinyl alcohol, double emulsion, solvent evaporation, sustained release, food industry, cosmetics.

Resumo

O setor de produtos cosméticos e de higiene pessoal é um mercado internacional com grande expansão económica. O interesse em produtos de valor acrescentado, naturais e biológicos na indústria cosmética e alimentar criou a necessidade de procurar novos produtos bem como a reformulação de outros com ingredientes biológicos e funcionais. Para além disso, no setor alimentar, os consumidores estão cada vez mais conscientes da relação entre a dieta alimentar e a saúde. Neste sentido, de maneira a ser eficaz nestas indústrias e evitar a sua competitividade, os produtos comercializados necessitam de ser diferenciados sendo que tal pode ser alcançado usando tecnologias emergentes como a microencapsulação. Por exemplo, os óleos essenciais, devido à sua volatilidade e baixa estabilidade, uma vez que são facilmente oxidados ou degradados na presença de oxigénio, luz, enzimas ou calor, durante o seu processamento, utilização e armazenamento, são normalmente encapsulados com o intuito de superar essas desvantagens. Existem inúmeras possibilidades de usar a microencapsulação, sendo que a técnica da dupla emulsão com evaporação do solvente foi o método utilizado no presente estudo.

O objetivo deste projeto é preparar e caracterizar micropartículas de eugenol pela técnica anteriormente referida, usando a etil celulose como agente encapsulante; bem como avaliar a influência de determinados parâmetros de formulação nas características das micropartículas finais. Também foi realizada a libertação sustentada do eugenol a partir de micropartículas de etilcelulose em quatro veículos diferentes: água ultrapura e octanol para simular veículos cosméticos hidrofílicos e lipofílicos, e meios ácidos e básicos para recriar os fluidos gástrico e intestinal. Objetivos específicos incluíram a validação do método analítico utilizado, bem a determinação de parâmetros de quantificação e de confiabilidade. Os principais resultados obtidos para o método analítico foram uma boa linearidade e boa precisão, além de baixos limites de deteção e de quantificação, para os quatro meios investigados. Quanto aos parâmetros de caracterização obtidos, o rendimento do produto variou entre 77.5 ± 6.1% e 82.4 ± 2.6%; a eficiência de encapsulação entre 76.4 ± 21.6% e 93.3 ± 0.2%; e o conteúdo de água variou entre 48.1 ± 7.2% e 87.5 ± 0.6%. As micropartículas preparadas apresentaram tamanhos variados entre 128 ± 0.2 µm e 850 ± 19.1 µm para as formulações globais testadas, sendo genericamente esféricas, polidispersas, porosas e superficialmente rugosas. As percentagens acumuladas das libertações mais altas após 24 horas foram 77% em água ultra pura, 68% no meio básico, 91% no meio ácido e 50% em octanol.

Palavras-chave: Microencapsulação, eugenol, etilcelulose, diclorometano, metanol, álcool polivinílico, dupla emulsão, evaporação do solvente, libertação sustentada, indústria alimentar, cosméticos.

Table of Contents

List of Figures ..... **iii**

List of Tables ..... **iv**

Glossary ..... **v**

1 INTRODUCTION **1**

1.1 Background **1**

1.2 Essential Oils **2**

1.2.1 Eugenol 3

1.2.2 Detection Methods 4

1.3 Microencapsulation **6**

1.3.1 Microencapsulation in Industry 8

1.3.2 Encapsulating Materials 9

1.3.3 Microencapsulation Techniques 12

1.4 Double Emulsion Solvent Evaporation Technique ($W_1/O/W_2$) **14**

1.4.1 Oil/Organic Phase (O) 16

1.4.2 External Aqueous Phase (W_2) – Continuous Phase (CP) 17

1.4.3 Parameters influencing microencapsulation 19

1.5 Controlled Release **21**

2 STATE OF THE ART **27**

3 WORK OUTLINE **33**

3.1 Aims of the thesis **33**

3.2 Thesis Organization **33**

4 MATERIALS AND METHODS **35**

4.1 MATERIALS **35**

4.1.1 Reagents and Chemicals 35

4.1.2 Equipments 35

4.2 METHODS **36**

4.2.1 Analytical Methodology for Eugenol Quantification 36

4.2.2 Preparation of eugenol microparticles by Double Emulsion Solvent Evaporation Technique 38

4.2.3 Characterization parameters of Eugenol Microparticles 40

4.2.4 Particle Morphology and Particle Size Distribution 41

4.2.5 Sustained Release studies 42
4.2.6 Quality Assurance and Control 42
4.2.7 Waste Treatment 42

5 RESULTS AND DISCUSSION 45

5.1 Analytical Method Validation 45

5.2 Microparticles Characterization 47

5.3 Particle Morphology and Particle Size Distribution 50

5.4 Sustained release studies 56

6 CONCLUSIONS 63

7 LIMITATIONS AND RECOMMENDATIONS FOR FUTURE WORK 65

References 67

Appendix I

Analytical Method Validation I

Microparticles Characterization III

Particle Morphology and Particle Size Distribution III

Sustained release studies V

Photographs of the Practical Procedure used in the Laboratory VI

List of Figures

Figure 1 – Eugenol essential oil extracted from clove bud oil (A) and other natural sources of this compound: cinnamon and anise (B) 4

Figure 2 – Schematic summary of the UV-Vis Spectrophotometry method 5

Figure 3 - Main types of internal morphologies for microparticles: mononuclear microcapsule (A); polynuclear microcapsule (B) matrix-type microsphere (C) (Adapted from Paulo and Santos, 2017) 7

Figure 4 – Structure of ethyl cellulose polymer 11

Figure 5 - Schematic overview of the principal process steps in microsphere preparation by double emulsion solvent extraction/evaporation technique (Adapted from Freitas et al. 2005) 15

Figure 6 - Different phases among different release profiles (Adapted from Fredenberg et al., 2011) 23

Figure 7 - Calibration curves of eugenol for the validation of the UV-Vis Spectrometry method in UPW, Basic Medium (pH 9), Acid Medium (pH 2) and OCT 45

Figure 8 - Particle morphology results obtained using a low level of magnification (320x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right) 52

Figure 9 - Particle morphology results obtained using a medium level of magnification (1000x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right) 52

Figure 10 - Particle morphology results obtained using a high level of magnification (2000x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right) 53

Figure 11 - Results of the sustained release study in 24 hours, at room temperature, in UPW (pH 5.6), for the four microparticles formulations 60

Figure 12 - Results of the sustained release study in 24 hours, at room temperature, in Basic Medium (pH 9), for the four microparticles formulations 60

Figure 13 - Results of the sustained release study in 24 hours, at room temperature, in Acid Medium (pH 2), for the four microparticles formulations 61

Figure 14 - Results of the sustained release study in 24 hours, at room temperature, in OCT, for the four microparticles formulations..... 61

List of Tables

Table 1 - Properties and chemical characteristics of eugenol compound.....4

Table 2 – Summary of different types of encapsulating materials used in microencapsulation studies 10

Table 3 - Different types of microencapsulation techniques and conventional methods reported for several microencapsulation studies in the past years 12

Table 4 - Main parameters, conditions and properties influencing microencapsulation..... 19

Table 5 – Main events responsible for the activation of the diffusion processes as well as main factors that could influence the release rate of encapsulated volatile essential oils..... 22

Table 6 – Literature overview about eugenol encapsulation in the recent years 30

Table 7 - Summary of the concentrations used for the preparation of the standard solutions of eugenol in all the four mediums investigated..... 36

Table 8 – Summary of the four formulations of eugenol microparticles performed for this project.... 39

Table 9 – Linearity parameters for the validation of the UV-Vis Spectrometry calibration curves for eugenol quantification in UPW, Basic Medium (pH 9), Acid Medium (pH 2), and OCT 46

Table 10 –Microparticles characterization parameters obtained for the four formulations 47

Table 11 – Particle mean diameter and polydispersitivity degree results for the four formulations ... 54

Glossary

(2-HP-β–CD) - 2hydroxypropyl - βcyclodextrin

(β-CD) - β–cyclodextrin

BSA - Bovine Serum Albumin

DCM - Dichloromethane

DP/CP – Dispersive Phase to Continuous Phase

EC – Ethyl Cellulose

EE - Encapsulation Efficiency

EOs – Essential Oils

EU - The European Union

EUG – Eugenol

F - Formulation

FDA - United States Food and Drug Administration

FID - Flame Ionization Detector

GA - Gum Arabic

GC - Gas Chromatography

GRAS - Generally Recognized As Safe

HLB - Hydrophilic-Lipophilic Balance

IR - Infrared

L-B-L - Layer-By-Layer

LLDPE - Linear Low Density Polyethylene

LOD – Limit of Detection

Log k_{ow} - Partition Coefficient

LOQ – Limit of Quantification

M - Mean

MS - Mass Spectrometry

M_w – Molecular Weight

n.a. – Not Applied

n.f. – Not Found

O – Organic/Oil Phase

OCT - Octanol

OSA - Octenyl Succinic Anhydride

O/W - Oil-in-Water

O/W/O - Oil-in-Water-in-Oil

OWR – Oil-Water Ratio

PACA – Polyalkylcyanoacrylate

PAMAM – Polyamidoamine

PCL - Polycaprolactone

PEG – Polyethyleneglycol

PGA – Polyglycolide

PGU - Purity Gum Ultra

PHB – Polyhydroxybutyrate

pKa - Acid Dissociation Constant

PLA - Polylactide Acid

PLGA - Polylactide Co-Glycolide Acid

PMMA – Polymethylacrylate

PMT - Photomultiplier Tube

PVA – Polyvinyl Alcohol

PVP – Polyvinylpyrrolidone

PY – Product Yield

RSD – Relative Standard Deviation

s – Standard Deviation

SEM - Scanning Electron Microscopy

SPAN – Polydispersity Degree

TEM - Transmission Electron Microscopy

UPW – Ultra-Pure Water

UV-Vis – Ultraviolet-Visible

W_1 – Internal Aqueous Phase

W_2 – External Aqueous Phase

WC – Water Content

W/O – Water-in-Oil

W/O/W – Water-in-Oil-in-Water

1 INTRODUCTION

1.1 Background

The increasing interest of consumers in natural and biological products created the demand for new valuable products as well as the reformulation of others with new or improved functionalities (Carvalho et al., 2016). Added-value products for cosmetic, personal care and food sectors have to be differentiate, which can be accomplished using innovative and promising technologies such as microencapsulation and nanoencapsulation technologies (Casanova and Santos, 2016; Hu et al., 2016). According to Paulo and Santos (2017), a clear distinction between nanoencapsulation and microencapsulation is not consensual among researchers. However, both industries apply these processes mainly for the protection of the core material from environment degradation (e.g. heat, moisture, air, light) and to study the controlled release of their compounds over time (Desai and Park, 2005). Therefore, these delivery systems ensure that the encapsulated material will reach the target within certain time without being adversely affected by the environment through which it passes (Champagne and Fustier, 2007; Dubey et al., 2009; Gouin, 2004; Hu et al., 2016). Although nanotechnology is a talented technology to successfully entrap bioactive compounds, microencapsulation is addressed in this study considering the final intended application of the microparticles obtained in this study (cosmetics/food). Moreover, nanotechnology has some important drawbacks not only due to the environmental problems associated to removal of nanoparticles acting as pollutants being able to adhere other compounds due to their small size (called nanowastes) (Pacheco-Torgal and Jalali, 2011), but also because nanoparticles could possibly pass directly into the bloodstream even before they are absorbed where they are needed (e.g. duodenum), as well as can react with elements that are already in the human body and generate toxins (Georgiana et al., 2014).

Furthermore, several active ingredients for cosmetic applications have been studied. Some examples are antioxidants, essential oils (EOs), fragrances, sun filters, anti-aging molecules, moisturisers, and tanning, whitening or lightening agents. Likewise, active ingredients for food applications can be vitamins, enzymes, flavours, minerals, or preservatives (Desai and Park, 2005) that usually have a sustained release in human body. In addition, EOs like eugenol are volatile compounds and usually present low stability since they are easily oxidized or decomposed in the presence of oxygen, light, enzymes or heat, during processing, storage or utilization phases (Goñi et al., 2016). Consequently, these compounds are microencapsulated in order to overcome these disadvantages and still keep their physicochemical properties (Carvalho et al., 2016; Dubey et al., 2009; Ghosh, 2006).

1.2 Essential Oils

The EOs have been used in several industrial sectors, such as in the agricultural, food, cosmetic, pharmaceutical and textile sectors, due to their antiviral, bactericidal, anti-parasitical, antimicrobial, insecticidal, and fungicidal activities against several bacterial strains (Ali et al., 2005; Devi et al., 2010; Schmidt et al., 2007). In addition, they are also used for medicinal applications due to various functional properties, such as antioxidant, organoleptic, nutritional, gastronomic, anti-inflammatory, anti-vasoconstrictive, anti-arrhythmic, antiulcer, antidiabetic, antithrombotic, antihypertension, anticancer, anti-aging, antidepressant, and antipyretic (Bakkali et al., 2008; Bakry et al., 2016).

Therefore, EOs are complex mixtures of liquid, natural, and volatile bioactive compounds accumulated in vesicles and existing in all different parts of the plants (leaves, seeds, fruits, beans, flowers, peel, roots, berries, and spikes), although they are mainly extracted from their leaves (Bakry et al., 2016). The same author suggested that EOs are produced by aromatic plants as secondary metabolites, being not directly related to growth and metabolism of the plant but having essential protective and defensive functions (Demain and Fang, 2000). Chemically, EOs are considered mixtures of terpenes, terpenoids, and phenylpropanoids, contributing to the taste and aroma of the plant. Phenylpropanoids comprise a six-carbon aromatic phenol group and a three-carbon side group, and are considered as a small part of the EOs, being the most studied: eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde (Pereira, 2007). Further, EOs are characterized by the compounds presented in large concentrations among their constitution and that determine their flavour, fragrance and biological properties. Several EOs have been increased their recognition among population, since they have been classified and generally recognized as safe (GRAS) food additives (Hu et al., 2016). Therefore, EOs at significantly low concentrations could prevent the development of pathogenic microorganisms in food products, acting as preservative agents and ensuring their conservation (Kamatou, Vermaak, and Viljoen, 2012). Additionally, in oral care products, it was already reported that EOs mouthwashes have similar efficacy against bacteria as alcohol commercial mouthwashes offering additional benefits such as reduce gingivitis and oral malodour, suggesting that natural EOs can be considered suitable substitutes of chemical and synthetic additives that may be dangerous for human health (Kamatou et al., 2012). Moreover, EOs can also be added as antiseptic and anti-inflammatory agents in anti-acne formulations in case of cosmetics or as local anaesthetic and analgesic in clinical dentistry, for example (Markowitz et al., 1992; Pereira, 2007). Furthermore, EOs are normally also used in cosmetic sector as fragrances, although some EOs could as well be used for relaxation and healing skin applications. Additionally, EOs that have antioxidant properties are used in sunscreens products to protect skin from oxidative stress promoted by sun radiation. Similarly, essential oils are also used in hair care products to provide conditioning and shine effects, and to protect and enhance the beauty of the scalp (Casanova and Santos, 2016).

Although there are several functional properties and bioactivities making EOs attractive for several industries, these oils are volatile, chemically unstable and susceptible to oxidative deterioration and decomposition in the presence of oxygen, light, enzymes, moisture or heat during processing, utilization and storage (Casanova and Santos, 2016). These disadvantages might have a negative impact on the shelf-stability, sensory perception, and the overall acceptance of the final products by the consumers. Therefore, since these chemical and physical effects could change the quality of the final products, some authors concluded that microencapsulation techniques may be the best answer to effectively minimize those problems by protecting the active ingredient (Carvalho et al., 2016; Dubey et al., 2009; Ghosh, 2006). Furthermore, microencapsulation approaches will limit the compounds degradation and loss during processing and storage as well as it will control the compound delivery at the desired time and location (Bakry et al., 2016).

1.2.1 Eugenol

The eugenol (EUG) is an oily liquid with a pleasant, floral and spicy odour, whereas when exposed to a natural environment can get darker due to oxidation and degradation. Chemically, it is a volatile phenolic compound (Goñi et al., 2016) that has low solubility in water but present solubility in organic solvents (Table 1). In addition, EUG is an aromatic and stimulating essential oil around the nature being used, for instance, as an aphrodisiac or anti-depressive. It could also be use in the form of infusion or powder for the treatment of vomiting, flatulence, or nausea, in the medical sector. Noteworthy, this oil was considered and approved as food additive by the United States Food and Drug Administration (FDA) in order to be further included in food products (Gong et al., 2016) as a flavouring or preservative agent. Therefore, it could be used as a preservative due to its antimicrobial, antibacterial and antioxidant applications in the field of food safety (Gomes et al., 2011; Hu et al., 2016; Hui et al., 2017; Siano et al., 2003).

Likewise, other authors already reported the benefits of this oil in several industries, such as food (Choi et al., 2009; Kayaci et al., 2013; Woranuch and Yoksan, 2013), clinical dentistry (Markowitz et al., 1992; Pereira, 2007), medicine and pharmaceutical (Ali et al., 2005; Capasso et al., 2000; Pereira et al., 2013; Devi et al., 2010; Hussain et al., 2011; Schmidt et al., 2007), cosmetic and perfumery as a fragrance enhancer in such as perfumes, soaps, shampoos, moisturizers, aftershave lotions, bath products, oral hygiene products, mouthwashes, hair care products, moisturizers and skin care products (Kamatou, Vermaak, and Viljoen, 2012; Markowitz et al., 1992; Pereira, 2007; Rastogi et al., 1998), as well as agricultural acting as a pesticide, insecticide or fungicide (Gong et al., 2016; Kamatou et al., 2012; Mazzafera, 2003).

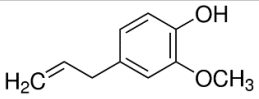
Moreover, it has unique characteristics as anaesthetic, analgesic, antiseptic, anti-ulcerogenic, anti-carcinogenic, anticonvulsant, anti-inflammatory, antifungal and antiviral

compound (Capasso et al., 2000; Goñi et al., 2016; Hussain et al., 2011; Kamatou et al., 2012; Pereira et al., 2013; Yogalakshmi et al., 2010).

Alternative EOs containing eugenol such as pimento oil (*Pimenta dioica)* and bay oil (*Pimenta racemose)* were investigated by Schmidt et al. (2007). However, eugenol is mainly extracted from clove (*Syzygium aromaticum)*, basil (*Ocimum basilicum*), nutmeg (*Myristica fragrans*), cinnamon (*Cinnamomum zeylanicum)*, and bay laurel (*Laurus nobilis*) leafs, being eugenol the major constituent of clove bud oil (> 80%) (Hu et al., 2016; Pereira, 2007). Therefore, the clove plant (*Syzygium aromaticum*) (Figure 1) has been cultivated since ancient times in India, Madagascar, Sri Lanka, South China and Indonesia (Pereira, 2007). However, it is produced and commercially exported worldwide, and used for several applications in traditional medicine, pharmaceuticals, food flavourings, food preservation, and cosmetic fragrances. This aromatic spice is used as seasoning in cooking due to its aroma and flavor, conferred by the volatile aromatic compound namely eugenol (Pereira, 2007).

Figure 1 - Eugenol essential oil extracted from clove bud oil (A) and other natural sources of this compound: cinnamon and anise (B)

Table 1 - Properties and chemical characteristics of eugenol compound

Compound [a]	Eugenol
IUPAC designation	4-allyl-2-methoxyphenol
Cas Number	97-53-0
Chemical Formula	$C_{10}H_{12}O_2$
Chemical Structure	
Molecular weight (M_w) (g/mol)	164.204
Boiling point (°C) (at 760 mmHg)	225
Water solubility at 25 °C (mg/L)	2460
UV range in water (nm)	290
Miscible with	Alcohol, Chloroform, Ether
Acid dissociation constant (pKa) (25 °C)	10.19

[a] *PubChem Compound Database, 2004*

1.2.2 Detection Methods

According to the literature there are several detection techniques used in chemical procedures. Since EOs are volatile compounds, in order to determine the chemical composition of the volatile fraction and identify their chemical compounds, EOs are normally analysed by

chromatography techniques, such as gas chromatography (GC) equipped with flame ionisation detector (FID) or gas chromatography coupled to mass spectrometry (MS), since these methods are more precise, comparing to spectrophotometric ones, and allow the temperature control. However, unlike most EOs, eugenol essential oil has singular structural properties since it has a benzene ring associated to a double bound which is normally associated to an increase in the stabilization of the compound among solutions, allowing the detection of the respective molecule by spectrophotometric methods. Therefore, the UV-Vis (ultraviolet-visible) spectrophotometer method is a viable method for the analysis of this compound (Figure 2).

Regarding this method, it is known that electromagnetic radiation is used to carry out the measurements (visible and ultra-violet spectral range between 190-900 nm). The minimum-wavelength limit was explained by Tissue (2012) due to the possible absorption of interferers at low ultraviolet wavelengths, such as atmospheric gases (<180 nm) or water (≈190 nm).

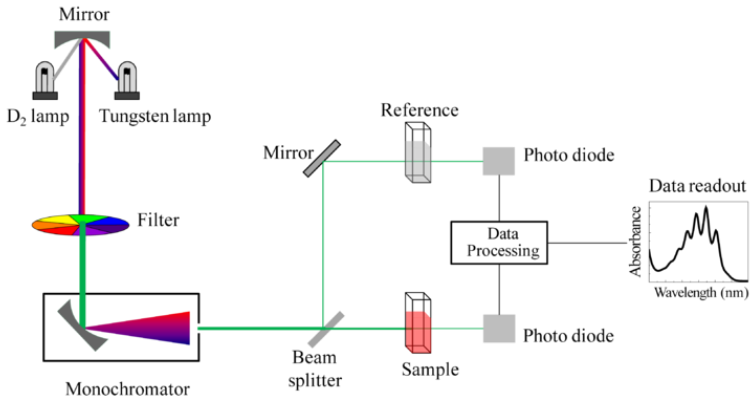


Figure 2 - Schematic summary of the UV-Vis Spectrophotometry method

This method is based on the usage of monochromatic light. Therefore, spectrometers typically use as a light source a tungsten-iodine/halogen light source for the near-UV to near infrared (IR) spectral range (e.g. 380 – 1000 nm) and a deuterium discharge lamp for the UV measurements from about 200 to 380 nm. Instruments with these lamps will combine the two light beams or automatically switch between the lamp beams using a flip mirror when scanning between the UV and visible regions (Mantele and Deniz, 2017; Tissue, 2012). Moreover, the diffraction by so-called “order filters” in the beam near the monochromator let the desired light pass and block or at least reduce the higher order light intensity. In addition, the holographic diffraction grating in a single or double monochromator or spectrograph will be responsible for the dispersion of the wavelengths of the continuum light sources. Furthermore, a light beam will be redirected automatically to the appropriate detector when scanning between the visible and near IR regions. Therefore, according to Tissue (2012), most of the commercialized UV-Vis spectrophotometers use one of the following optical designs: (a) single beam where a single light beam passes through a sample holder to a single detector; or (b) scanning dual beam where the

light beam alternately passes through sample and reference holders to a single detector as the spectrometer scans each wavelength. Additionally, there is a detector, which in a single-detector instrument is a photodiode, phototube, or photomultiplier tube (PMT), but the dual-beam UV/Vis – near IR spectrophotometers utilize the combination of a PMT with an infrared detector.

Nevertheless, some traces of unwanted light in the measuring beam limit the application of the Lambert-Beer's law (Equation 1), which is responsible fot the obtaining of the absorbance numerical value (A) associated to UV-VIS Spectroscopy experiment.

$$A = \varepsilon \times c \times d \quad \text{(Equation 1)}$$

Where ε is the molar absorption coefficient, c the concentration and d the path length of the measuring beam in the sample (Mantele and Deniz, 2017).

Most of the UV-Vis spectrophotometers contain a 10- to 200-mm wide sample compartment through which the light beam passes, normally holding liquid sample quartz cuvettes with a 10mm path length. Moreover, most of the modern UV-Vis spectrophotometers are controlled by an integrated microprocessor or a software program running on a separate computer (Tissue, 2012).

1.3 Microencapsulation

In 1953, Green and Schleicher published a patent about the production of microencapsulated dyes by complex coacervation technique using gelatin and gum arabic (GA) for the manufacture of carbonless copying paper. In the 1960s, microencapsulation of cholesteric liquid crystal by complex coacervation of gelatin and acacia was reported to produce a thermosensitive display material. Further, since the mid of 1970s, microencapsulation has gained popularity in pharmaceutical industry as well as for many other applications in daily use (Dubey et al., 2009). Therefore, after the first applications, this technology has been widely studied and used for several purposes, such as cosmetics and food industries.

Microencapsulation is generally defined as a group of techniques in which micron-sized liquid droplets, solid particles or gas compounds (core) are enclosed. This internal part contains the active ingredient (e.g., a hardener, a biocide, an essential oil, an antioxidant, etc.), while the external part comprises the encapsulating material (shell), protecting and isolating the core permanently or temporarily from the exterior. Depending on the final application, several types of core materials could be encapsulated (Ghosh, 2006). Normally, microparticles have spherical shape and small diameters ranging between 1 and 1000 μm. In fact, this property is relevant about microencapsulation because the typical smaller size of the particles allows the increase of the surface area, make it available for several reactions. Although nano-scale particles have a

bigger surface area (smaller size) comparatively to micron-scale particles, it was already reported the drawbacks that nano-systems have in the Background sub-chapter (Georgiana et al., 2014; Pacheco-Torgal and Jalali, 2011).

Therefore, micron-sized particles can be classified based on their internal structure and morphology. The entrapped compounds could be located in an inner cavity totally involved and surrounded by the encapsulating material like a reservoir system (microcapsules), or embedded in a matrix system dispersed in the polymer (microspheres) as it is demonstrated in Figure 3 (Ghosh, 2006; Stella et al., 2017). Therefore, the reservoir systems can be distinguished as a mononuclear/monocored or polynuclear/polycored microcapsules (Dubey et al., 2009; Ghosh, 2006) (Figure 3). Mononuclear microcapsules contain the shell around the core and have a single hollow chamber within the capsule. Polynuclear microcapsules have many cores enclosed within their respective shell, being heterogeneous dispersed and surrounded by another encapsulating material (Dubey et al., 2009; Ghosh, 2006). In addition to these three basic morphologies, microcapsules can also be mononuclear with multiple shells (Ghosh, 2006).

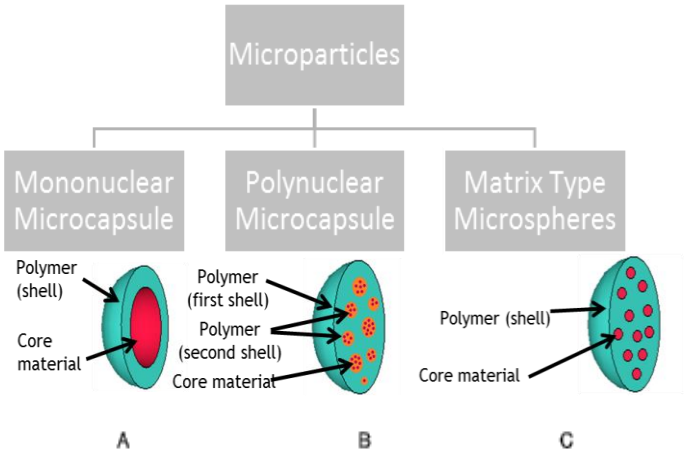


Figure 3 - Main types of internal morphologies for microparticles: mononuclear microcapsule (A); polynuclear microcapsule (B) matrix-type microsphere (C) (Adapted from Paulo and Santos, 2017)

protect sensitive, reactive or unstable materials from the external environment, preventing the degradation and decomposition from enzymatic occurrences, physico-chemical influences (e.g. from pH, light, temperature, moisture), or from reactions like oxidation and dehydration; (b) for purposes of controlled or sustained release of the encapsulated compounds; (c) to improve the solubility, flowability and dispersibility of the molecules; (d) to separate incompatible components for functional reasons; (e) to give the possibility of combining properties of different types of materials (e.g. organic and inorganic); (f) to mask undesired properties of the active components (such as odour, taste and activity); and (g) to allow safe and convenient handling of toxic materials (Dubey et al., 2009; Ghosh, 2006).

It is well described that the size, structure and shape of the microparticles are dependent of the materials and processes involved in the development and production of the final microparticles (Estevinho et al., 2013; Pereira, 2007). Therefore, these parameters influence

the encapsulation efficiency (EE), the product yield (PY), and the water content (WC) in the microparticles, as well as their controlled or sustained release capacity. Moreover, according to Casanova and Santos (2016), the content of core material that is effectively encapsulated depends on several variables such as shell material properties, the chosen microencapsulation technique, and the chemical nature of the core (molecular weight, chemical functionality, polarity, volatility). In addition, compatibility of the core material with the shell is an important criterion related with the increased efficiency of the microencapsulation (Carvalho et al., 2016; Casanova and Santos, 2016; Ghosh, 2006).

1.3.1 Microencapsulation in Industry

There are numerous possibilities and alternatives to use microencapsulation techniques to obtain added-value products. Several industrial sectors were already reported due to the exploration of this technology, such as pharmaceutical (68%), food (13%), cosmetic (8%), textile (5%), biomedical (3%), agricultural (2%), and electronic (1%) industries (Ghosh, 2006; Martins et al., 2014; Paulo and Santos, 2017). According to Gomes et al. (2011), micron-sized carriers have been applied with relevance either in scientific or industrial field.

Besides that, it is known that consumers are looking for effective, natural and safe ingredients that are able to contribute for their health, beauty and wellness. For instance, cosmetic, perfumery and personal care industry have been investing in emergent, distinctive and innovative technologies, like microencapsulation, as well as in scientific knowledge, to deliver into the global market new or reformulated products that fits the increasingly demanding of expectations by the consumers. Therefore, microencapsulation has been gaining interest in the global cosmetic market, being applied in balms, shampoos, skin creams and lotions, bath gels, hair products, sunscreens, soaps, makeup, toothpastes, and perfumes (Casanova and Santos, 2016). In addition, according to Finch and Bodmeier (2000), microencapsulation could be used in the manufacture of beauty aids, such as perfumes for a delayed release or cosmetic formulations like make-up removers. Furthermore, microencapsulation techniques have been widely used in the development of other products in order to increase the stability and the effectiveness as well as to improve their sensory properties increasing the overall business applications.

Another example is the food industry (Desai and Park, 2005; Finch and Bodmeier, 2000; Silva et al., 2014). For instance, in the current industrialised world consumers are increasingly aware of the relationship between diet and health. Thus, the demand of functional food products that contributes for a balanced diet (Sagalowicz and Leser, 2010), includes the growing interest of consumers about the benefits that certain ingredients have. Additionally, according to Silva et al. (2014), microencapsulation helps the creation of foods that are not only a source of nutrients but are also associated with the health of consumers. For instance, it is possible to encapsulate compounds that increase the level of calcium to prevent osteoporosis, or create additives of phenolic compounds to prevent heart problems. However, the development of

functional foods through the addition of bioactive ingredients to improve their nutritional value holds several technological challenges and usually compromise their taste, colour, texture and aroma (Champagne and Fustier, 2007). Furthermore, sometimes they degrade and become hazardous by oxidation reactions or react with other food components or external environmental factors limiting the bioavailability of the total food system (Dubey et al., 2009; Finch and Bodmeier, 2000; Gouin, 2004; Pereira, 2007). Other authors, Sagalowicz and Leser (2010), also suggested that several micronutrients are hardly soluble in aqueous systems revealing an incompatibility between the active ingredient and the food matrix, limited stability against chemical or physical degradation, as well as uncontrolled release or bioavailability. Therefore, micron-size delivery systems have been largely used due to their capability to carry-on an antimicrobial agent from the polymeric matrix to the liquid-like food. This allow to reach a determined concentration of the core ingredient in the food during a specific period of time (Mastromatteo et al., 2009).

Nonetheless, this technique allows the incorporation of minerals, vitamins, preservatives and essential oils in food products, as well as nutrients to mask taste, or flavours to protect their volatile nature during storage (Desai and Park, 2005). Additionally, microencapsulation was reported by Champagne and Fustier (2007) as a useful tool to also deliver other bioactive compounds into foods formulations, such as probiotics, fatty acids and antioxidants. Therefore, a wide range of delivery systems is available for their use in food systems, such as powder particles, single and multiple emulsions, molecular complexes, liposomes and vesicles (Sagalowicz and Leser, 2010). Moreover, the increasing interest in active food packaging also allowed the usage of microencapsulation for the creation of systems that are able to control the microbiological deterioration of fresh food products. In addition, according to Finch and Bodmeier (2000) many foods and animal feed additives could also be encapsulated in order to improve life storage by reducing volatilization and oxidative degradation. Consequently, to find the appropriate delivery system is especially challenging in the food industry compared to other industrial fields.

Some other examples of microencapsulation application procedures are: (a) detergent industry that use shell wall dissolution of powder detergents for release of encapsulated protease enzymes in order to remove bloodstains from the clothing, (b) microcapsules may be also used in the formulation of laundry products, fabric softeners, and brighteners; and (c) agriculture, where pesticides or repellents are microencapsulated to be released over time, allowing farmers to apply these products less often (Finch and Bodmeier, 2000).

1.3.2 Encapsulating Materials

While choosing the encapsulating agent some parameters should be considered, namely: its toxicity, stability, viscosity, biocompatibility and mechanical properties; the intended surface morphology and particle size of the final microparticles; the compatibility between the active

ingredient and the wall material; and the processing and economic factors involved. Although the necessary properties of the shell material are dependent of final application, there are some general characteristics that should be accomplished by the encapsulating agent (Desai and Park, 2005), since it will be crucial for the process efficiency, the stability of the final microparticles, and for the degree of protection of the core. Therefore, the easy workability during encapsulation, to be chemically non-reactive with the core material, to be inexpensive, the capability to completely release the core material, the solubility in the solvents used, and the ability to give the maximum protection of the active ingredient against the environmental conditions, are other parameters that need to be considered.

Commonly, the shell materials used in the production of microencapsulated ingredients are divided into: (a) natural polymers such as polysaccharides and sugars, animal and plant derived proteins, or lipids; (b) synthetic polymers; and (c) inorganic materials, being these ones also used as second polymers or simply additives (Casanova and Santos, 2016; Desai and Park, 2005; Martins et al., 2014). In addition, encapsulation in polymeric particles may prevent the active ingredient from degradation, control the rate and extent of release, and deliver a higher concentration of the active ingredient to the desired site, lowering the possible toxic effects (Stella et al., 2017). Polymers used as matrix for delivery systems could be divided according to their compatibility (biocompatible or non-biocompatible), degradability (biodegradable or non-biodegradable) and source (natural, synthetic, semi-synthetic) (Tiwari and Prerana, 2011). Thus, encapsulating agents can be selected from a wide variety of organic or inorganic materials as it is shown in Table 2.

Table 2 - Summary of different types of encapsulating materials used in microencapsulation studies

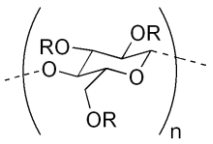
Type of polymer	Source of the polymer	Class	Examples	References
Organic	Natural	Lipids	Waxes and fats; Paraffin; Oils; Glycerides; Phospholipids; Carotenoids.	Desai and Park, 2005
		Animal and Plant derived Proteins	Gluten; Gelatine; Casein; Albumin; Peptides; Soy proteins.	
		Polysaccharides and Sugars	Starches; Modified starch; Chitosan; Modified chitosan; Cyclodextrins; Maltodextrins; Alginates; Sodium caseinate; Pectin; Dextran; Gum arabic; Agar; Carrageenan; Ethyl cellulose; Carboximethylcellulose.	
	Synthetic	Polyesters	PGA; PAMAM; PMMA; PACA; PEG; PLA; PLGA; PCL; PVA; PVP.	Stella et al., 2017
Inorganic	n.a.	n.a.	Silicates; Clays; Polyphosphates.	

n.a. - Not applied; PGA - Polyglycolide; PAMAM - Polyamidoamine; PMMA - Polymethylacrylate; PACA - Polyalkylcyanoacrylate; PEG - Polyethyleneglycol; PLA - Polylacticacid; PLGA - Polylactic co-glycolic acid; PCL - Polycaprolactone; PVA - Polyvinyl alcohol; PVP - Polyvinylpyrrolidone.

The most commonly used polymers to produce particles include polylacticacid (PLA), polyglycolide (PGA), polylactic co-glycolic acid (PLGA), ethyl cellulose (EC), cellulose acetate

phthalate, polycaprolactone (PCL), polyhydroxybutyrate (PHB), and polyalkylcyanoacrylate (PACA) (Dubey et al., 2009; Stella et al., 2017). For instance, PCL, polymethylacrylate (PMMA) or PLGA polymers are normally used not only for improving the long-term stability and solubility of the core material in cosmetic formulations, but also for enhancing and prolonging the effectiveness of the active ingredients. Additionally, PLA and PLGA are the most widely used in microencapsulation due to its excellent biocompatibility properties (Tiwari and Prerana, 2011). Moreover, PACA polymers were reported in medical sector and polyamidoamine (PAMAM) polymers were reported for bio-applications (Ammala, 2013).

Furthermore, ethyl cellulose is a derivative of cellulose in which some of the hydroxyl groups on the repeating glucose units could be converted into ethyl ether groups (Figure 4). It is classified as a semi-synthetic, non-biodegradable and biocompatible polymer. Therefore, this polymer is one of the most studied encapsulating agents for controlled release studies (Patil et al., 2016; Prasertmanakit et al., 2009; Voncina et al., 2009), being effective for protection of microcapsules from the environmental conditions of the stomach, whilst allowing active ingredient release in the intestinal tract to make it available for absorption (Prasertmanakit et al., 2009). This polymer was approved and accepted by both the FDA and The European Union (EU) for food, cosmetic and pharmaceutical applications (Eltayeb et al., 2015). Additionally, this polymer may act as an intestinal fibre (Cummings, 1984).



R = H or CH_2CH_3

Figure 4 - Structure of ethyl cellulose polymer

Moreover, among the numerous materials that have been used for the encapsulation, chitosan has been also pointed out, as well as gum arabic has been described as one of the most frequently used wall material in the microencapsulation for food industry. Alternatively, some encapsulating materials may be combined, once most of them do not have all the required properties when used alone (Abla and Banga, 2013; Estevinho et al., 2013). Thus, either encapsulating materials are employed combined with each other or there is the possibility of the addition of modifiers such as antioxidants, chelating agents, and surfactants (Desai and Park, 2005). Some examples of these mentioned combinations are: xanthan gum arabic/albumin, gelatin/gum arabic, and soybean protein isolate/pectin complexes (Jun-xia et al., 2011), as well as gelatin/pectin, whey protein/gum arabic, gelatin/acacia-gum, β-lactoglobulin/acacia gum, gelatin/heparin, gelatin/carboxymethyl guar gum, and albumin/alginic acid (Sagiri et al., 2015). Therefore, these mixtures of protein and polysaccharide molecules are normally used for flavour microencapsulation in food industry to improve the emulsifying properties. Furthermore, it is

important to consider that some properties of polymers, such as their degradation rate or mechanical properties, are strongly defined by their structural characteristics, including their molecular weight, composition, or nature of the chain end groups. Therefore, according to Ammala (2013), some polymers could be chemically functionalized and modified in order to obtain improved properties, allowing that those modified shell materials will exhibit better physical and mechanical properties when compared to the individual materials (Desai and Park, 2005).

Finally, it is important to consider that the composition of the shell material is not only one of the main critical parameters that determine the functional, compositional and morphological properties of the final microparticles, but also a parameter that will influence the final application performance of a particular encapsulated ingredient (Bakry et al., 2016; Desai and Park, 2005; Dubey et al., 2009).

1.3.3 Microencapsulation Techniques

As it is shown in Table 3, it is summarized the most conventional methods which have been reported and discussed in several microencapsulation studies (Bakry et al., 2016; Carvalho et al., 2016; Desai and Park, 2005; Dubey et al., 2009; Ghosh, 2006; Martins et al., 2014).

Table 3 - Different types of microencapsulation techniques and conventional methods reported for several microencapsulation studies in the past years

Microencapsulation Technique	Methods	References
Chemical	Dispersion polymerization; Interfacial polycondensation.	Ghosh, 2006
Physico-Chemical	Single or double emulsion with removal of solvent; Coacervation.	Bakry et al., 2016; Gouin, 2004.
Physico-Mechanical	Spray-drying; Extrusion.	

Microencapsulation techniques may be divided into two categories: (1) chemical techniques includes those methods where physical equipment is needed, but the chemical reactions involved are the controlling step; and (2) physical techniques comprise all the methods where physical equipment are necessarily required, being the hardening and the production of the microparticles the dominant steps, although chemical reactions can also be involved (Dubey et al., 2009).

Within the physical-mechanical techniques, the spray-drying technique is the most common microencapsulation method because it is simple, has a low commercial process cost, and allows the obtaining of typically monodisperse systems. It is mostly used for the encapsulation of pigments, lipids and many flavours (Brannon-Peppas, 1993; Estevinho et al., 2013; Ghosh, 2006). Despite all the advantages of this method, spray-drying technique has as major limitation the fact that for thermo-sensitives products such essential oils, this method

could be limited because the required high temperature cause volatilization and/or degradation of the product, according to Silva et al. (2014).

Among the physical-chemical techniques, there are the single and double emulsions techniques. Emulsification may be applied for microencapsulation of bioactive ingredients for several purposes in aqueous solutions, in order to form powders after emulsification. Therefore, emulsification technology can be considered one of the most important steps on the microencapsulation of essential oils. Moreover, an emulsion consists in at least two immiscible liquids, usually oil (O) and water (W), with one of the liquids being dispersed as small spherical droplets in the other liquid. A system that consists of oil droplets dispersed in an aqueous phase is called a simple oil-in-water (O/W) emulsion, whereas a system that consists of water droplets dispersed in an oil phase is called a simple water-in-oil (W/O) emulsion (Bakry et al., 2016). Furthermore, simple emulsification W/O with solvent evaporation was the first method proposed for the preparation of polymer particles. It is commonly described as a method where is possible to form an emulsion which allows the dissolution of the polymer solution in a volatile organic solvent (Bakry et al., 2016; Sagiri et al., 2015), where the active ingredient will be dispersed. Consequently, the entire mixture is heated under agitation in order to favour the evaporation of the solvent and the phase separation of the polymer should occurs, i.e., the encapsulating material makes a shell around the core material and encapsulates it. Due to the fact that some ingredients are thermo-sensitives and the boiling point of them is not desired, evaporation of the solvent could occur by continuous stirring at room temperature.

Along with the methods summarized in Table 3, several alternative methods were also reported for application in micro-sized products and formulations for numerous industries, such as: supercritical fluid technology, fluidized-bed coating, spray cooling/chilling, molecular inclusion, lyophilisation, layer-by-layer (L-B-L) assembly, sol-gel encapsulation, centrifugal techniques, electrostatic encapsulation, polymer precipitation, spinning disc, and crystallization (Brannon-Peppas, 1993; Dubey et al., 2009; Ghosh, 2006; Silva et al., 2014).

Despite the variety of available methods for encapsulation approaches no single process is suitable for all core materials or product applications. Therefore, the choice of the microencapsulation method for a particular process will depend on the several features, such as: (a) the final product expected application (b) the size, biodegradability and biocompatibility of the final microparticles; (c) the physico-chemical properties of the core material; (d) the proposed controlled release mechanism; (e) the production scale and the costs along the operation and manufacturing processes; and (f) the nature of the polymeric material used as encapsulating agent (Casanova and Santos, 2016; Ghosh, 2006; Shahidi et al.,1993). According to Stella et al. (2017), in order to select the appropriate technique, the physicochemical characteristics and properties of both the polymer and the active ingredient should be taken into account to ensure good interactions between the internal molecule and the outside matrix.

Finally, the microencapsulation method should normally be reproducible, simple, fast, effective and easy to implement at industrial scale (Martins et al., 2014). In fact, one of the most important aspects of microencapsulation for the industry it is the easy scaling-up of the methods used. Furthermore, in order to make some methods transition from the laboratory scale to the industrial scale, it is crucial to understand how the different process parameters and variables could affect the characteristics of the final microparticles (Carvalho et al., 2016).

1.4 Double Emulsion Solvent Evaporation Technique ($W_1/O/W_2$)

Microencapsulation has been widely explored by several industries, and although several encapsulation methods have been described (Dubey et al., 2009; Ghosh, 2006; Gouin, 2004), the double emulsion solvent evaporation was already applied and reported as an important microencapsulation technique (Zakeri-Milani et al., 2013). In fact, multiple emulsions like double emulsions have been developed in order to increase the encapsulation efficiency (Iqbal et al., 2015; Tiwari and Prerana, 2011) because it allows a better stability and a better entrapment of the molecules comparing to other incorporation methods (Lamprecht et al., 2000). One of the most common types of multiple emulsions is water-in-oil-in-water (W/O/W) double emulsion (Yan et al., 2013), which have received considerable attention as they are promising systems for drug delivery, as well as industrial applications in the areas of cosmetics (Miyazawa et al., 2000) and in foods (Matsumoto, Kita, and Yonezawa, 1978). Therefore, it is possible to consider two common types of multiple emulsions, the oil-in-water-in-oil (O/W/O) and W/O/W emulsion.

Further, since several hydrophilic compounds cannot be encapsulated into the carriers due to the rapid active ingredients partitioning and release into the external aqueous phase using single emulsions, consequently, many common compounds have been successfully encapsulated in microparticles by double emulsion techniques either with evaporation, extraction or diffusion of the solvent, such as drugs, proteins, vitamins, or cosmetic agents, essential oils (Akhtar and Yazan, 2008; Bitar et al., 2015; Cohen-Sela et al., 2009; Iqbal et al., 2015; Meng et al., 2004; Yener and Öke, 2009; Zakeri-Milani et al., 2013), for further incorporation in pharmaceutical, food or cosmetics (e.g. skin care) products.

In general, double emulsion technique is a versatile technique widely used for the preparation of biodegradable and non-biodegradable polymeric microparticles, as well as for the microencapsulation of a various types of cores materials. One the one hand, the main advantages of this approach are: the relative ease process of preparation, the potential to encapsulate both hydrophobic as well as hydrophilic molecules by W/O/W or O/W/O, respectively (such as drugs, cosmetics, foods and other high value products), the high encapsulation efficiency, the good product yield, the reproducibility, the possibility of prolonged release systems, the taste and odour masking, and the protection of the active ingredients not

only from the external environment but also from gastrointestinal conditions (Bakry et al., 2016; Sagiri et al., 2015; Stella et al., 2017). Despite that advantages, it was also suggested that: the physical instability when exposed to drying, heating, freezing, pH differences or high mineral concentrations conditions; the limited control release; the production of large, polydisperse and non-uniform particles; the fact that this technique is a two-step process (bi-phasic); and the difficulty to scale up this method, are considered the main relevant drawbacks of this method (Bakry et al., 2016; Sagiri et al., 2015; Stella et al., 2017). Furthermore, the fact that a long process of homogenization with high shear mixing force is required to obtain small emulsion droplets, which could damage the biomolecules and lead to a loss of bioactivity, was also suggested as a limitation of this technique according to the previous authors.

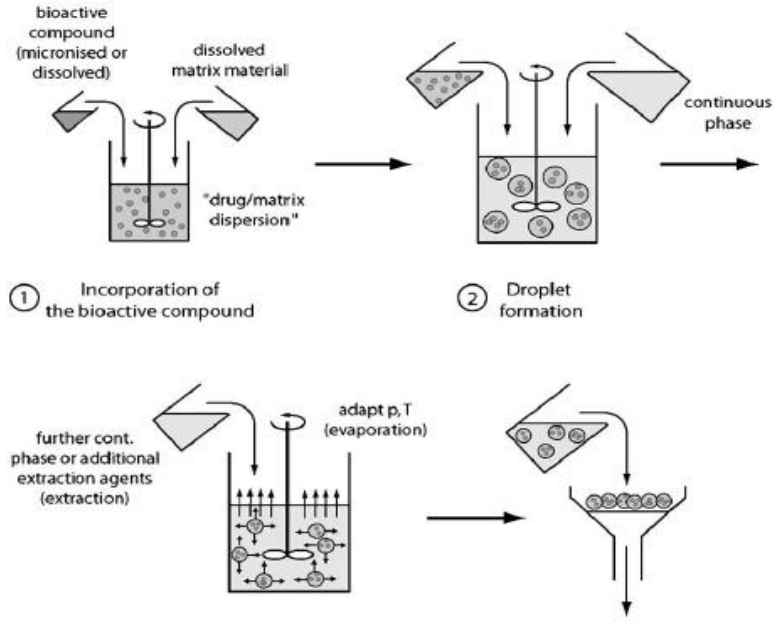


Figure 5 - Schematic overview of the principal process steps in microsphere preparation by double emulsion solvent extraction/evaporation technique (Adapted from Freitas et al. 2005)

Double emulsion technique ($W_1/O/W_2$) comprises four mainly steps (Figure 5). Firstly, the active ingredient is added to the inner aqueous phase (W_1) of the emulsion, and then it is dispersed into the oil phase that contains the polymer used (e.g. ethyl cellulose) dissolved in a volatile water immiscible organic solvent, such as dichloromethane (DCM) or chloroform, to form the first emulsion (W_1/O). Additionally, it is also possible to add water miscible co-solvents like methanol or acetone into the organic phase to help the dissolution of the active ingredient. Further, in the second step, the primary emulsion is added and dispersed into the outer aqueous phase (W_2) containing a specific surfactant with emulsifying and stabilising properties, such as poly(vinylalcohol) (PVA) or poly(vinylpyrrolidone) (PVP), to form the second emulsion ($W_1/O/W_2$), and, consequently, the small polymer droplets containing the encapsulated material (Casanova and Santos, 2016). The emulsions between the oil, the water and the emulsifier are normally prepared using a device called homogenizer, such as high shear mixer, high-pressure homogenizer, colloid mill, sonicator, or membrane homogenizer (Bakry et al., 2016).

Afterwards, the formation of the particulate dispersion is followed by the hardening of the droplets in order to produce the corresponding final polymer microparticles, either by solvent removal through solvent evaporation (by heat or under reduced pressure), or by solvent extraction into the continuous phase (with a third liquid that is a precipitant for the polymer and miscible with both water and solvent) (Freitas et al., 2005), causing polymer precipitation and particles formation (Stella et al., 2017). Additionally, it is known that is possible to remove the solvent also by diffusion (Meng et al., 2004). The main difference between emulsification evaporation method and emulsification diffusion technique is the type of solvent (immiscible vs partially miscible in water) and the higher stress applied into the emulsion (Stella et al., 2017). However, it is expected that evaporation of the solvent technique allows a slower solvent removal and a consequent better morphology of the final microparticles.

1.4.1 Oil/Organic Phase (O)

Regarding the encapsulating agent, the usage of different types of polymers in microparticles have received substantial interest in order to help active ingredients release in delivery systems. Therefore, the use of ethyl cellulose-based microparticles has been already studied for the encapsulation of essential oils using emulsion solvent evaporation technique (Patil et al., 2016), as well as it was also reported for the encapsulation of different cores using various methods (Eltayeb et al., 2015; Voncina et al., 2009).

According to Prasertmanakit et al. (2009), the concentration of EC polymer acting as the hydrophobic polymer responsible for the wall formation could be an interesting parameter to analyse due to the fact that it would have an effect on the results obtained for the characterization of the microparticles. Likewise, the concentration of EC in the dispersive phase exerted a significant impact into the entire microencapsulation process. In addition, some studies already used EC as encapsulating material obtaining different results related to microparticles characterization parameters (Eltayeb et al., 2015; Patil et al., 2016; Prasertmanakit et al., 2009; Voncina et al., 2009), suggesting that the reasons for this fact is the variety of core materials and encapsulation methods used.

Furthermore, in the dispersive phase, together with the encapsulating agent, the organic solvents are also added. Thus, the choice of the organic solvent is a crucial factor to prepare microparticles and to develop successful formulations. Therefore, some parameters should be considered when choosing an organic solvent, such as the ability of the solvent to dissolve the polymer and the active ingredient, its toxicity, and its miscibility with water. Therefore, it is preferred to choose a solvent with low toxicity, low boiling point and with low solubility in the continuous phase. According to Birnbaum et al. (2000), lower solubility of the organic solvent in water allows the creation of a more stable emulsion that leads to higher quality microparticles (i.e., spherical particles with nonporous surfaces and better size distributions). Additionally, the

capability of the solvent to dissolve large quantities of polymer makes it easier to control particle size distribution and encapsulation efficiency of the core material.

There are numerous common organic solvents used in the preparation of polymeric microparticles, such as chloroform, ethyl formate, DCM, or ethyl acetate. Ethyl acetate has limited solubility in water (slightly soluble). Then, it is necessary to consider that morphology of the final microparticles may be affected. However, once it is consider to be less toxic that DCM, ethyl acetate is used due less toxicity concerns (Stella et al., 2017). Additionally, the use of partially water-miscible organic solvent like ethyl acetate enable rapid diffusion through the aqueous phase forming smaller particles (Cohen-Sela et al., 2009). However, the use of DCM is more prevalent due to high ability to dissolve large amounts of the most common used polymers, making easier to control the particle size distribution (Birnbaum et al., 2000).

Nevertheless, DCM is toxic (suspected carcinogen and mutagen) and, consequently, a removal step of this solvent would be required during microencapsulation process. Moreover, this solvent is extremely low soluble in water and, therefore, it is usual to add water miscible co-solvents into the organic phase to help the dissolution of the active ingredient. This will facilitate the diffusion of water into dispersive phase and also to limit the toxicity factor, being methanol (MeOH) one of the most co-solvent reported for microencapsulation purposes (Tiwari and Prerana, 2011). Additionally, was also reported that this mixture (DCM/MeOH) favors the polymer precipitation (low solubility of the polymer in the dispersive phase) and improves the diffusion of the active ingredient from the internal aqueous phase to the external aqueous phase, allowing a higher encapsulation efficiency (Birnbaum et al., 2000). Finally, Cohen-Sela et al. (2009) and Birnbaum et al. (2000) suggested that combining the double emulsion system with a partially water-soluble organic solvent could allow the creation of more stable emulsions which will lead to microparticles with higher quality and improved formulation characteristics, such as smaller particles size, nonporous surfaces, lower size distributions, and spherical morphology.

1.4.2 External Aqueous Phase (W_2) – Continuous Phase (CP)

According to Stella et al. (2017), the fact that hydrophobic and hydrophilic molecules can be encapsulated using the double emulsion solvent evaporation technique created the necessity of addition and usage of suitable stabilizers in the external aqueous phase in order to maintain the stability of the microparticles, which are thermodynamically unstable systems. In fact, emulsifiers and surfactants are crucial for the production and protection of oil droplets by lowering the interfacial tension between the external aqueous phase and the oil phase (Hu et al., 2016; Leimann et al., 2009), avoiding the coalescence of the microparticles (Gomes et al., 2011).

Using this technique, the stability of the primary emulsion is a critical factor for the efficient encapsulation of the active ingredient (Yeo and Park, 2004). This could be explained

because the internal aqueous phase (W_1) tends to merge with the external aqueous phase (W_2). Some studies have been carried-out in order to overcome such limitation. For instance, Sagiri et al. (2015) concluded that by adding complex forming or gelling agents into the internal phase the emulsion would increase its stability. Yeo and Park (2004) concluded that the stability of the primary emulsion could be enhanced by including emulsifying agents such as bovine serum albumin (BSA), PVA, polysorbate (Tween-80 as a commercial designation), or sorbitan monooleate (Span-80 as a commercial designation), either in the internal aqueous phase (W_1) or in the external aqueous phase (W_2). Moreover, in order to obtain a kinetically stable solution, emulsifiers are added in the global emulsion system (Bakry et al., 2016).

According to Stella et al. (2017), polyesters are normally used and added as stabilizers for this technique, such as PLA, PLGA, PCL, PVP and PVA. Moreover, not only in this technique but also in other encapsulation methods, environmentally-friendly polymers such as PVA are preferable compared with traditional petroleum-based polymers such as polypropylene (Wen et al., 2016). Therefore, PVA is a hydrophilic, highly biocompatible and non-toxic polymer, which has been widely applied (Wen et al., 2016). It is also defined as a white coloured granular powder without taste, slightly soluble in ethanol, but insoluble in other organic solvents. The PVA was first prepared by Hermann and Haehnel in 1924 by hydrolysing polyvinyl acetate in ethanol with potassium hydroxide, although it is known that this polymer could be also produced commercially from polyvinyl acetate by a continuous process (Leimann et al., 2009). This compound is described has having its melting point at 180 to 190 °C and a degree of hydrolysis of 86.5 to 89% (Saxena, 2004).

In cosmetics and personal care industry, PVA may act as a viscosity increasing agent in aqueous solutions intended for cosmetic purposes. It is widely applied in formulations for eye make-up and skin care products (Bergfeld et al., 2013). According to the same author, the maximum concentration of use of PVA in this type of formulations is reported to be 25% (w/w).

In food industry, it is known that PVA could be used in several products, such as food supplements, tablets or capsules, and ready-to-eat products. The amount of PVA incorporated in the food products is limited and regulated by FDA (Borzelleca, Glinsmann, and Munro, 2002). Moreover, due to its viscosity, PVA could be used as a wall-forming material in microparticles and a moisture barrier film for foods that contain inclusions of active ingredients, which need to be protected from several environmental components, while simultaneously masking their taste and odour, as well as retain the overall texture, integrity and quality of the foods (Saxena, 2004).

In addition, the physical characteristics of PVA and its specific functional uses will be dependent on the degree of polymerization and the degree of hydrolysis, in order to understand how easy or faster the solubilisation of the polymer in water could be. Thus, PVA could be classified into two categories, namely: partially hydrolysed and fully hydrolysed, being the fully hydrolysed one the category mostly used for food applications. Moreover, this polymer is also

interesting because of it is ease of processing, its relatively simple chemical structure, and its potential use in pharmaceutical and biomedical fields (Leimann et al., 2009). Furthermore, the products in which this polymer is applied, normally have neutral pH and are stored at low or room temperature, assuring that breakdown or instability of PVA will not occur, which possibly would allow the creation of undesirable interactions between the polymer and the food constituents (Saxena, 2004). It is also known that this polymer also allow the handling of the final products and facilitates the possible ingestion and swallowing.

The oil or water affinity that is referred as hydrophilic-lipophilic balance (HLB) in surfactants is analogous to particle wettability characterized via the contact angle according to Marefati et al. (2015). Therefore, the HLB of a surfactant is defined as a measure of the degree to which it is hydrophilic or lipophilic, characterizing the final surfactant function. Moreover, this scale plays a crucial role for determining the surfactant-polymer interactions and controlling the porosity and roughness of the microparticles (Ortega-Oller et al., 2015). It is expected that PVA acts as an oil-in-water (O/W) emulsifying agent according to HLB scale, since it is a hydrophilic surfactant (HLB value in a range between 8 and 16). However, Turk et al. (2014) reported that PVA has a HLB value of 18, being considered a solubilizing agent.

Finally, it is important to consider that this polymer solution is crucial for microencapsulation approaches, since PVA is responsible for the system stabilization preventing the particles aggregation.

1.4.3 Parameters influencing microencapsulation

There are several factors that can affect the final microencapsulated products and their respective properties. According to Paulo and Santos (2017), these parameters could be divided into three main classes as described in Table 4.

Table 4 - Main parameters, conditions and properties influencing microencapsulation

Parameters	Variables	References
Formulation parameters	Viscosity Use of additives or surfactants Type of solvent used Oil-Water Ratio	Belščak-Cvitanović et al., 2011; Birnbaum et al., 2000; Kim et al., 2010.
Operating conditions	Temperature Stirring rate	Das and Rao, 2007; Piletti et al., 2017.
Properties of materials	Wall material and core material properties	Hojjati et al., 2011; Majeed et al., 2016; Sharif et al., 2016; Shulkin and Stöver, 2002.

Specifically, in the double emulsion-solvent evaporation method, the emulsification and stabilization of the droplets are considered as highly important factors. Therefore, some studies investigated the parameters and factors which affected the performance of this technique and, consequently, the resulting microparticles characteristics (Nihant et al., 1994; Meng et al., 2003; Tiwari and Prerana, 2011). For instance, Tiwari and Prerana (2011) investigated the type

and molecular weight of the polymer, the core material/polymer ratio, the solubility of the core material in continuous phase, the rate of solvent removal, the external aqueous phase volume, and the interactions between the active ingredient/polymer, as process variables important to study in solvent evaporation methods. Another important parameter that should be considered is the internal aqueous phase volume or, alternatively, the oil-water ratio (OWR). Similarly, the influence of solvent removal method and wall polymer composition on microspheres characteristics in $W_1/O/W_2$ double emulsion technique was studied by Meng et al. (2004). It was also found that the volume of the outer aqueous phase (W_2) in the second emulsification step and the shear stress in the solidification step had a significant effect on the diffusion rate of the solvent from the droplets into the outer aqueous solution, and thereby on the characteristics of the resultant microparticles (Tao et al., 2003). The influence of the homogenization time as well as the amount of surfactant in the external aqueous phase were already reported by Lamprecht et al. (2000) as parameters which could influence microparticles prepared by double emulsion pressure homogenization technique in their size and dispersity. In another study, according to Stella et al. (2017), particle size could also be affected by the forces and temperature applied during the emulsion, the polymer concentration and molecular weight, as well as the concentration and type of stabilizers used. Some other parameters were as well investigated by Iqbal et al. (2015) in order to obtain an optimized homogeneous droplet size, such as dispersive phase to continuous phase (DP/CP) ratio and types of solvents. Moreover, the influence of stirring rate and oil volume fraction on the microparticle size distribution was already evaluated (Leimann et al., 2009). Besides that, in double emulsion method, other experimental variables such as the solvent removal method and the internal aqueous phase volume were investigated for particle size and polydispersity degree results, in a study conducted by Zambaux et al. (1998). Therefore, the physical characteristics of the particles could be mainly affected by the technique used, as well as molecular mass and the addition of surfactants or other additives (Gomes et al., 2011).

Therefore, the overall microparticles characteristics that may be affected by these parameters are: water absorption, product yield, encapsulation efficiency (Jafari et al., 2008), active ingredient release rate (Thonggoom et al., 2016), microparticles surface morphology, mean particle size, and particle size distribution. For instance, according to Bakry et al (2016), the size and the shape of the final microparticles will depend on the wall materials and the methods used to prepare them. In addition, in another study, Voncina et al. (2009) showed that the morphology (degree of sphericity), the size of the microparticles and the encapsulation efficiency, could also be influence by the stirring speed employed during the microencapsulation process. Other studies (Jyothi et al., 2010; Yeo and Park, 2004), analysed the main parameters that could affect the encapsulation efficiency, such as concentration of the polymer, ratio between DP/CP, solubility of the polymer in the organic solvent, solubility of the solvent in water, rate of solvent removal, stability of the primary emulsion, and the molecular weight of

the polymer. Besides that, these authors also suggested that whenever the previous formulation parameters contribute to a faster solidification of the microparticles, the encapsulation efficiency of them would be higher. Additionally, it is important to consider that the rate of solvent removal will influence not only the solidification rate of the dispersive phase but also the morphology of the final microparticles and, subsequently, their porosity (Mehta et al. 1994). However, according to Jyothi et al. (2010), the rate of solvent removal could be adjusted by the initial range of temperature evaporation or by the final volume of the dilution medium.

Finally, was recognized that the stability of the primary emulsion have an important effect on the results obtained among microencapsulation studies which use double emulsion solvent evaporation technique. Meng et al. (2003) suggested that fast diffusion rate of the internal aqueous phase through the oil phase until the external aqueous phase would favour the polymer precipitation, allowing a rapid solidification of the microparticles and, consequently, an increase in the encapsulation efficiency results.

1.5 Controlled Release

Controlled release could be defined as a physical-chemical phenomenon in which one or more encapsulated active ingredients are isolated from the external environment and make available when the release is desired, under the influence of a specific stimulus at a recommended rate, a desired place and a certain time (Desai and Park, 2005; Martins et al., 2014). This is a crucial behaviour in microencapsulation approaches, which will not only improve the success of the encapsulation procedure of several compounds as well as the expansion of their numerous possible applications. Indeed, controlled release technology could be used to increase the encapsulation efficiency of many ingredients with improved efficacy, safety and convenience (Bakry et al., 2016; Casanova and Santos, 2016; Pereira, 2007). Noteworthy, the same authors suggested that the study of controlled release mechanisms helps to overcome the loss of activity of the encapsulated ingredients, as well as the unsuccessful utilization of them.

Along with controlled released, there also is the sustained release, normally defined as one mode of controlled release systems that allows the delivery of a constant concentration of active compounds at the final targets. In fact, controlled release is defined as the release where the active ingredient concentration is the same every time, while sustained release is defined as the release where the time interval of release is the same every time. Thus, normally, at initial stage, the amount of released oil is large and then slowly decreases and becomes constant. In this case, the release of microparticles content is done by diffusional processes with erosion of the polymeric matrix, being the release rate often mentioned as diffusion-controlled at initial stage while at final stage period it is known as degradation/erosion-controlled release (Fredenberg et al., 2011).

The release of the core material from delivery systems could be classified based on different mechanisms, such as: (a) transport through water-filled pores; (b) rupture of the wall material under influence of mechanical or osmotic forces (osmotic pumping) with degradation of the polymer chains (forced convection); (c) erosion by melting or dissolution of the encapsulating polymer (without transport); (d) transport or diffusion through the wall material (Dubey et al., 2009; Fredenberg et al., 2011; Martins et al., 2014). According to Gomes et al., (2011), the general mechanisms by which active ingredients could be released from a delivery system are the combination of the previous events.

Moreover, Casanova and Santos (2016), Desai and Park (2005), Dubey et al. (2009), and Ghosh (2006) suggested that any event could be used to activate the release of the encapsulated ingredient by diffusional processes, as well as there are several factors that could also influence the release rate of encapsulated volatile essential oils (Table 5), leading either to the erosion of the polymeric matrix creating channels on the surface of the particles, or to a further hydrolysis process of the ester bonds of the polymer creating micro channels inside the particles allowing the release of the active ingredients from the core to the external environment.

Table 5 - Main events responsible for the activation of the diffusion processes as well as main factors that could influence the release rate of encapsulated volatile essential oils

Events activating diffusion	Factors influencing release rate	References
Enzymatic activity Use of solvents Biodegradation Osmotic force pH change Mechanical stress (outside pressure) Temperature Light Time	Properties of the particle (size, porosity, shape) Component materials of microparticles Ratio between the core and wall material Method of encapsulation Medium of release Environmental factors (temperature, pH, stirring, osmolality, humidity) Properties of the polymer (water-uptake, chain length, flexibility, molecular weight, plasticization extent, thickness, pore size) Polymer-core interactions Pore closure Polymer-environment interactions Swelling degree (absorption of fluids)	Casanova and Santos, 2016; Desai and Park, 2005; Dubey et al., 2009; Fredenberg et al., 2011; Ghosh, 2006; Patil et al., 2016.

Furthermore, Peppas and Ende (1997) studied the release of different essential oils including eugenol, concluding that the amount of essential oil release was correlated to the thermodynamic compatibility of the oil–polymer pair, i.e., was dependent on its temperature, solubility parameter, and chemical structure. In addition, the release rate of encapsulated flavour compounds was also studied by other authors, which concluded that release rate depend on several processes, such as shape and geometry of the carriers structure, diffusion of the active ingredient compound through the matrix, particles size and composition, processing conditions used, and degradation/dissolution of the carriers (Eltayeb et al., 2015; Seo et al., 2010).

Regarding the release profiles, several phases and profiles have been described (Fredenberg et al., 2011), such as zero-order release, bi- or tri-phasic profiles, burst phases and second phases, being all shown in Figure 6. Although not all the release profiles follow the traditional tri-phasic release profile, this is the most common, comprising: (a) Phase I - typically described as a burst release (or a fast release) attributed to non-encapsulated core on the surface or active ingredient molecules close to the surface easily accessible by hydration, as well as the formation of cracks or holes leading to particles disintegration; (b) Phase II - is known as slow release diffusional phase where are few existing pores or the polymer is excessively dense; and (c) Phase III - usually described as a faster release profile (second burst) due to hydration or degradation and erosion of the polymer. According to Yeo and Park (2004), there are some formulation parameters that may cause and influence the initial burst release, such as molecular weight, composition of the continuous phase, concentration and hydrophilicity of the polymer, as well as the distribution of the active ingredient in microparticles. Additionally, the release profiles of encapsulated compounds could be affected by the method of encapsulation, the release medium, the pH and by the interactions between the core material, the encapsulating agent and auxiliary ingredients added. Therefore, all of these parameters should be optimized to obtain an optimum controlled and sustained release system.

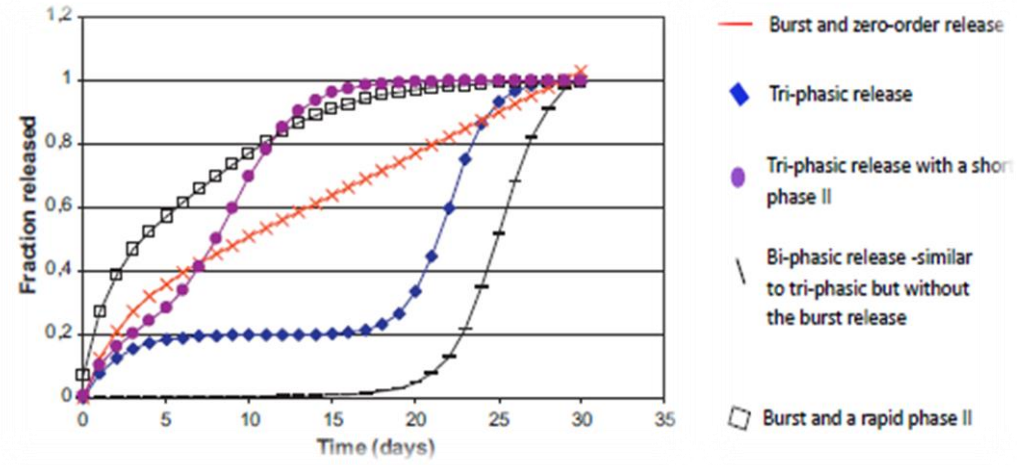


Figure 6 - Different phases among different release profiles (Adapted from Fredenberg et al., 2011)

In the present study, a special focus was made for both cosmetic and food industries. Once, it is known that the excess release of essential oils in cosmetic or food formulations could enhance unpleasant smell and, the controlled release is expected to overcome this situation. In cosmetic industry, commonly in skin delivery systems, the microparticle could break due to pressure release mechanism applied by friction against the skin, and subsequently removed from the body through normal metabolic pathways. However, other compounds could potentially accumulate in body tissues and cause irritation. Therefore, it is required that non-toxic and safe materials are used in order to reach the target within the specific layer of skin. However, some

active cosmetic substances are too hydrophilic to pass through the *stratum corneum* or too lipophilic to partition into the epidermis. Additionally, the compound needs to guarantee the lipophilic or hydrophilic characteristics that allow its solubilisation in the cosmetic itself, as well as to ensure its stability during formulation, storage and application. Additionally, Peppas and Ende (1997) investigated the usage of pH-triggered devices for fragrance release in contact with the skin, for personal care and consumer products.

Furthermore, depending on the application intended, the active ingredient may be released on the *stratum corneum* or in a specific skin layer (Casanova and Santos, 2016). According to Maghraby et al., (2008), the main mechanisms that have been investigated for particles acting as skin delivery systems of bioactive compounds are: (a) permeation of the core material freely in the skin from microparticles adsorbed in the *stratum corneum*; (b) vesicle adsorption to *stratum corneum* surface with subsequent transfer of the compound directly from particles to skin; (c) vesicle fusion and mixture with the *stratum corneum* matrix increasing the release of the active ingredient in the skin; and (d) intact vesicular skin penetration mechanism either by traditional or deformable vesicles. Additionally, the efficacy of topically applied active ingredients in cosmetic industry will be dependent on the type of vehicle used for the delivery, protection and release, as well as on the design of the appropriate carriers. Some critical aspects should be considered when delivering a cosmetic active ingredient through the skin, such as: (a) the right concentration of the components; (b) the formulation type; (c) right site of action of the cosmetic ingredient; (d) particle size; and (e) the correct application time of the product on the skin (Casanova and Santos, 2016).

Likewise, in food industry, the controlled release normally acts through some medium *stimuli*, such as heating, temperature or pH variations, leading to dissolution, diffusion, erosion, biodegradation or fracture of the microparticle. Therefore, several mechanisms of active material release have been proposed (Pereira, 2007). Generally, the release of the encapsulated ingredient depends on the geometry of the particle and the properties of the encapsulating agent used. Furthermore, it was suggested that microencapsulation technologies allow the delivery of bioactive compounds from food products into the gastrointestinal tract (Champagne and Fustier, 2007), being that EUG was released from glassy polymers slowly, although a complete release at 30 ºC was achieved after 13 hours (Peppas and Ende, 1997). This compound was also investigated by several authors in order to prove it proimising biological properties against several microorganisms (Ali et al., 2005; Devi et al., 2010; Gomes et al., 2011; Hui et al., 2017; Pereira et al., 2013; Schmidt et al., 2007), which are present in the human gastric tract and, therefore, the activity of eugenol at low pH levels let Ali et al. (2005) to suggest that it efficacy in an environment such as human stomach should be investigated and achieved. Moreover, Majeed et al. (2016) observed lower absorption of essential oil in upper gastrointestinal tract which was mostly released in the small intestine resulting in enhanced bactericidal efficacy of eugenol.

One of the main concerns of this approach is the fact that compounds like aromas are normally released quickly and effectively when food comes into contact with the mouth. Thus, solid microcapsules or powders cannot be simply added to an aqueous food product without losing the barrier and stabilization function of the solid capsule shell material. Therefore, if this type of release is intended, it is necessary to ensure that wall materials used are generally water-soluble, such as starch and dextrin. However, other molecules such as vitamins should just be released after ingestion, either in the stomach or the intestine. Thus, it is usual to use hydrophobic materials such as waxes, cellulose derivatives or proteins in this cases avoiding a possible dissolution of the polymer into the aqueous phase, releasing the ingredients before the intended time and site (Azeredo, 2005). As a result, delivering active ingredients in a liquid matrix requires the use of different encapsulation and protection strategies (Sagalowicz and Leser, 2010).

2 STATE OF THE ART

Although in the past decades many essential oils have been investigated and analysed towards their application in several industrial formulations and products, the literature research for this project is focused on studies where eugenol essentiol oil was studied, encapsulated and released in recent years. Table 6 summarizes the overall information collected.

Recently, Piletti et al. (2017) investigated the encapsulation process of eugenol in β-cyclodextrin (β–CD), in order to obtain a structure that is not toxic and it is resistant to high temperature treatments. The results suggested that within a thermal treatment at a temperature of 80 °C for 2 h, the complex exhibited significant antibacterial action against *Escherichia coli* and *Staphylococcus aureus*, which confirmed the thermal protection and stability of the eugenol molecules. The authors used a temperature higher than the volatilization temperature of eugenol because they were expecting that the encapsulated fraction was thermally protected in the complex being only released at higher temperatures. Therefore, the main conclusion was that encapsulation of eugenol molecules by β–CD could be an excellent method for protecting and preserving the antibacterial action of EUG-β–CD from the effects of thermal degradation, supporting the idea of application of these complexes as antimicrobial additives in materials that require thermal processing or in environments with higher temperatures.

Moreover, according to Woranuch and Yoksan (2013), it was also possible to improve the thermal stability of eugenol by encapsulating this molecule into chitosan nanoparticles via an emulsion–ionic gelation crosslinking method. This property was verified through encapsulated eugenol by extrusion at 155 ◦C with a model plastic. The results suggested that eugenol-loaded chitosan nanoparticles could possibly be used as antioxidants for various thermal processing applications, including bioactive plastics for food packaging. In a study designed by Goñi et al. (2016), linear low density polyethylene (LLDPE) films were impregnated with eugenol using CO_2 as the solvent and super-critical impregnation technique as a method, in order to obtain a loaded material to be also applied in active food packaging. Thus, impregnation runs were performed in a lab-scale high pressure stirred cell at 45 ºC during 4h, and the effect of pressure and depressurization rate on impregnation yield was evaluated. Moreover, it was suggested that the main plasticizing effect is due to supercritical CO_2, and was also observed that at 15 MPa and slow pressure release, the polymer swelling and plasticization was higher, allowing a better penetration of eugenol. Furthermore, similarly as Piletti et al. (2017), Kayaci et al. (2013) also investigated the use of cyclodextrins for microencapsulation. This author conducted a study where eugenol-cyclodextrin inclusion complexes (EUG/CD-IC) were encapsulated in PVA nanofibers via electrospinning technique in order to achieve high thermal stability and slow release of the molecule. Three types of cyclodextrins (α-CD, β-CD, and γ-CD) were used in order

to find out the most favourable CD type for the stabilization of eugenol. The authors concluded that the approach used in this study could also be useful for application in food industry and active food packaging, due to the extremely large surface area of nanofibers along with specific functionality that enhances thermal stability and durability as well as slows release of the active compound by CD inclusion complexation. In addition, higher thermal stability, enhanced durability and slower release of EUG was observed for PVA/EUG/γ-CDIC indicating that γ-CD was the most suitable type of CD to be the host for EUG.

Some other authors concluded that the hydrophobicity of eugenol and trans-cinnamaldehyde could be a limitation for their use within the food industry. Therefore, spherical PLGA nanoparticles with entrapped eugenol and trans-cinnamaldehyde were synthesized aiming the production of possible antimicrobial delivery systems. Controlled release experiments were conducted *in vitro* at 37 ºC and 100 rpm for 72 hours, and the results showed an initial burst followed by a slower and continuous rate of release of the antimicrobial entrapped inside the PLGA matrix. Finally, these nanoparticles proved to be efficient in inhibiting growth of bacteria strains like *Salmonella* spp. and *Listeria* spp., allowing the authors to suggest that the application of these antimicrobial nanoparticles in food systems could be effective for inhibiting the growing process of these specific pathogens, showing again the great potential in the design of new active food packaging (Gomes et al., 2011), as described elsewhere (Goñi et al., 2016; Kayaci et al., 2013; Woranuch and Yoksan, 2013).

In another study, similarly as Kayaci et al.(2013) and Piletti et al. (2017), Gong et al. (2016) investigated the complex inclusion of eugenol into βCD. Additionally, this author studied its structural characterization, antifungal activity, and its mode of action for the control of *Peronophythora litchii* in postharvest fresh litchi fruits. Therefore, *in vitro* assays showed that βCD–eugenol significantly inhibited *Peronophythora litchii* colony growth. The authors suggested that the antifungal activity of β–CD–eugenol showed great potential as a controlled-release agent against these microorganisms, leading towards the main conclusion that this complex seems to be a promising candidate as a safe and highly effective fungicidal microcapsule (Gong et al., 2016). Furthermore, Hu et al. (2016) studied the development of eugenol oil nanoemulsions using gum arabic and lecithin as natural food grade emulsifiers, as well as the study of their antimicrobial capacity. In addition, this study also investigated different drying techniques as a parameter of influence on the morphology and redispersibility of nanoemulsion powders. The main conclusion was that spray-drying equipment allowed to obtain smaller particles when compared to the ones obtained by freeze-drying. However, it is important to consider that in this study, eugenol oil was premixed with ethanol (used as a co-surfactant), which played a critical role in reducing the mean particle size and improved the morphology and uniformity of the powders during spray drying. In conclusion, the antimicrobial activity of eugenol against *Listeria monocytogenes* and *Salmonella Enteritidis* may lead towards numerous

potential applications of eugenol nanoemulsions as a preservative or sanitizer in the food industry. Nonetheless, Majeed et al. (2016) suggested that the use of eugenol in food, beverage or pharmaceutical industries was limited due to its poor solubility and low availability. Therefore, they investigated the influence of several parameters on *in vitro* lipid digestion as well as the controlled release of eugenol from nanoparticles using different types of emulsions with different droplet diameters. The results obtained showed that the mean particle size of the emulsions have progressively increased as they passed through the simulated gastrointestinal tract (mouth, stomach and intestine phases), which was explained due to flocculation, droplet coalescence, and digestion. Moreover, it was also concluded that an increase in percentage of eugenol release was associated with a decrease in the droplet diameter that was higher on the emulsions which contained large hydrophobic cores that better solubilized lipophilic eugenol molecule.

Additionally, Seo et al. (2010) supported the idea that was possible to investigate the thermo-physical properties of eugenol encapsulated with β-CD by molecular inclusion, like some other authors already suggested (Gong et al., 2016; Kayaci et al., 2013; Piletti et al., 2017). In this study, the eugenol storage and release characteristics using different relative humidity and storage temperatures were investigated. The main result highlighted that the release rate increased with increasing relative humidity and storage temperature. Given that, the authors concluded that high water absorption promoted eugenol release rate, resulting in a dissociation of the β–CD as a host from the βCD-eugenol complex (Seo et al., 2010). In another study, Choi et al. (2009) also studied the encapsulation of eugenol by inclusion method with β–CD and 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD), as well as by an emulsion–diffusion method with PCL. The study of oxidation stability revealed the emulsion–diffusion method was more efficient than the molecular inclusion one. Therefore, the authors concluded that the emulsion–diffusion method was the most effective for eugenol encapsulation due to higher protection from light oxidation during storage time because the complete wrapping of eugenol by PCL layer, when compared to inclusion method. In addition, it was suggested that β–CD was more effective than 2-HP-β-CD for eugenol encapsulation because the side chain of hydroxypropyl group of 2-HP-β-CD might interrupt eugenol inclusion within the cavity of the cyclodextrin molecule.

Finally, some other researches also addressed the encapsulation of clove oil and its bio-compounds such as eugenol (Cortés-Rojas, 2015; Meneses, 2016; Pereira, 2007).

Table 6 - Literature overview about eugenol encapsulation in the recent years

Encapsulating Material and Method	Aims	Results	References
Complex inclusion in βCD	Study of antibacterial properties of the complex; Production of a structure resistant to temperature treatments.	Thermal protection of eugenol molecules in the complex; Antibacterial action against *Escherichia coli* and *Staphylococcus aureus*; Mean size: 12 μm.	Piletti et al. (2017)
LLDPE films impregnated with eugenol using CO_2 by super-critical impregnation technique	Study of mechanical properties like elongation capacity and tensile strength; Antioxidant capacity of the films for application in active food packaging; Effect of pressure and depressurization rate on impregnation yield.	Distribution of eugenol not totally homogeneous; Decrease in the crystallinity degree after high pressure treatment; High yield values at low depressurization rate conditions;	Goñi et al. (2016)
Complex inclusion in βCD	Structural characterization, antifungal activity, and mode of action for the control of *Peronophythora litchii* in postharvest fresh litchi fruits.	Disruptive effects of the complex on the morphology and ultrastructure of the microorganism; Inhibition of colony growth (antifungal activity of the complex); Potential as a safe and highly effective fungicidal microcapsule.	Gong et al. (2016)
Nanoemulsions using GA and lecithin as natural food grade emulsifiers	Study of the antimicrobial activity; Influence of drying techniques on the morphology and redispersibility of nanoemulsion powders.	Spherical powders with a size of less than 500 nm obtained by spray-drying equipment; Antimicrobial activity against *Listeria monocytogenes* and *Salmonella Enteritidis;* Applications as a food preservative or sanitizer.	Hu et al. (2016)
Nanoemulsions emulsified by OSA modified starch PGU	Influence of particle size and carrier oil type, on *in vitro* lipid digestion; Study of controlled release of eugenol using different types of oil-in-water emulsions with different droplet diameters.	Mean particle size progressively increased as they passed through the simulated gastrointestinal tract; Increase in percentage of release of eugenol explained by a decrease in the droplet diameter; Faster release rate on *in vitro* lipid digestion due to a bigger exposure of the droplets to the lipase enzyme.	Majeed et al. (2016)
Chitosan nanoparticles via an emulsion–ionic gelation crosslinking method	Study of thermal stability of eugenol for various thermal processing applications.	Nanoparticles could be used as antioxidants for bioactive plastics for food packaging; EE in the ranges of 1.1–20.2%; Spherical shape with 80–100 nm as particle diameter.	Woranuch and Yoksan (2013)

Table 6 - Literature overview about eugenol encapsulation in the recent years (Cont.)

EUG/CD-IC encapsulated in PVA nanofibers via electrospinning technique	Study three types of CD to achieve high thermal stability and slow release of the molecule, for application in active food packaging.	The size/shape fit between the host CD and guest EUG molecule is an important factor for forming inclusion complexation; γ-CD was the most suitable type of CD; EUG was not complexed within PVA/EUG α-CD nanofibers due to the small cavity size; Slower release of EUG was achieved for PVA/EUG/β-CD-IC and PVA/EUG/γ-CD-IC nanofibers due to inclusion complexation.	Kayaci et al. (2013)
Spherical PLGA nanoparticles entrapped eugenol and *trans*-cinnamaldehyde by emulsion evaporation method using PVA	Create delivery systems for future antimicrobial applications in food industry.	Efficient in inhibiting growth of bacteria strains like *Salmonella* spp. and *Listeria* spp.; Potential in the design of new active food packaging; Entrapment efficiency for eugenol and *trans* cinnamaldehyde was 98 and 92%, respectively; Mean size: 200 nm; In 72 hours, 64 and 87% was released for eugenol and *trans*-cinnamaldehyde, respectively.	Gomes et al. (2011)
β–CD by molecular inclusion	Study thermo-physical properties of eugenol; Study eugenol storage and release characteristics using different relative humidity and storage temperatures.	Mean size: 340 nm; 91.7% of inclusion efficiency; Increased release rate with increasing relative humidity and storage temperature.	Seo et al. (2010)
β–CD and 2-HP-β-CD by inclusion method, and PCL particles by emulsion-diffusion method	Most effective method for eugenol encapsulation to protect it from light oxidation during storage time.	Mean sizes: 320 nm; Polydisperse size distributions in inclusion method compared to emulsion-diffusion method; EE: 100, 90.9 and 89.1% for PCL particles, β-CD and 2-HP-β-CD complexes, respectively; Emulsion-diffusion method is more efficient.	Choi, et al. (2009)

LLDPE - Linear Low Density Polyethylene; OSA - Octenyl Succinic Anhydride; PGU - Purity Gum Ultra; PLGA - Polylactic co-glycolic acid; PVA - Poly (vinyl alcohol); GA - Gum Arabic; β-CD - β-cyclodextrin; 2-HP-β-CD - 2-hydroxypropyl-β-cyclodextrin; PCL - Polycaprolactone; EUG/CD-IC - Eugenol-cyclodextrin inclusion complexes; EE - Encapsulation Eficciency.

3 WORK OUTLINE

3.1 Aims of the thesis

The purpose of this study is to prepare and characterize eugenol-loaded ethyl cellulose microparticles by double emulsion solvent evaporation technique. In this study is also pretended to accomplish the release studies of eugenol in four different mediums: ultra-pure water (UPW) at pH 5.6 and octanol (OCT) in order to recreate hydrophilic and lipophilic cosmetic vehicles, and acid and basic mediums to recreate the gastro and intestinal fluids. Furthermore, the influence of selected formulation parameters on the final characteristics of the microparticles (encapsulation efficiency, product yield, water content, morphology and size) was studied through four microencapsulation formulations (F1, F2, F3, F4) in triplicate. In addition specific aims were set, such as: (1) to develop and validate the analytical method (UV-Vis Spectrophotometry) for eugenol determination and quantification; (2) to determine performance parameters such as quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision).

3.2 Thesis Organization

This master thesis is divided and organized in six chapters and respective subchapters: introduction, state of the art, work outline, materials and methods, results and discussion, and conclusion. Other sections such as the limitations and future work, the references used and an appendix are also presented. Firstly, in Chapter 1, a background section is presented, where a general introduction of the thesis topic is outlined and the main reasons are identified of why this is a worthwhile question. In the same chapter, in another section, a descriptive review about theoretical concepts such as essential oils, microencapsulation techniques and applications, encapsulating materials, parameters influencing microencapsulation, and sustained release theory, are presented. Properties and applications of eugenol are also discussed in this section. In Chapter 2, is provided a review of the state of the art relevant to this work, namely the encapsulation, and release of eugenol. In Chapter 3 it is presented the aims of the thesis and the overall organization of the thesis. In Chapter 4 it is described the materials and methods used for the development, characterization and analysis of the microparticles. In chapter 5 it is presented all the results as well as the respective discussion, and the main conclusions are presented in Chapter 6.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Reagents and Chemicals

Analytical Eugenol (Ref. E-5504, CAS 97-53-0) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), as well as methanol (Ref. 32213, CAS 67-56-1). Dichloromethane (Ref. 32222, CAS 75-09-2) was purchased from Honeywell Riedel-de-Haën™ (Seelze, Germany). Polyvinyl alcohol (Ref. P8136-250G, 87-90% hydrolysed, (M_w) = 30.000–70.000, CAS 9002-89-5) and ethyl cellulose (Ref. 433837-250G, Viscosity 46 cP, CAS 9004-57-3) were purchased in Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Octanol with a purity of 99% (Ref. A15977, CAS 111-87-5) was purchased from Alfa Aesar (Haverhill, MA, EUA). Prepared solutions of hydrochloric acid and sodium bicarbonate were obtained in the laboratory. Ultra-pure water was obtained in the laboratory using a Merck Millipore Milli-Q water purification equipment (Billerica, MA, USA). All the reagents used were of analytical grade purity.

4.1.2 Equipments

All weight measurements were performed using a Mettler Toldedo AG245 analytical balance (Columbus, OH, USA). The preparation of the organic solution for microencapsulation was performed using an ultrasonic bath (P Selecta, Barcelona, Spain). During microencapsulation technique, the mixture of the aqueous phase and the organic phase was carried-out using a high-performance homogenizer (IKA T18 Digital ULTRA-TURRAX ®, Staufen, Germany). All the agitation steps were accomplished using a vortex shaker (IKA VORTEX GENIUS 3, Staufen, Germany) at 230 V, and an AREX Digital stirring plate (VELP Scientifica, Monza, Italy). To obtain the microparticles, a vacuum filtration system (KNF Neuberger, Breisgau, Germany) and a G4 filter were used. In order to obtain the final dried microparticles, the microparticles suspension were freeze-dried using a bench top VirTis freeze-dryer (SP Scientific, NY, USA). Detection and quantification of eugenol, as well as the validation of the analytical methods and the sustained release assessments were performed by UV-Vis Spectrophotometry analysis, using a V-530 (JASCO, OK, USA), a PU 8625 (Philips, Amsterdam, Netherlands) and a 6305 (JENWAY, Staffordshire, UK) UV-Vis Spectrophotometers. For the sustained release study, a Multi Stirrer stirring plate (VELP Scientifica, Monza, Italy) was used. To prepare the acid and the basic mediums, pH measurements were performed using a SensION™ + PH3 (Hach-Lange, CO, USA). For microparticles characterization, particle morphology was assessed using a bench-top Scanning Electron Microscopy (SEM) equipment, and the particle size distribution was evaluated by Laser Granulometry technique using a Particle Size Distribution Analyser named Coulter Counter-LS 230 (Coulter Corporation, FL, USA). In order to obtain the encapsulation efficiency, a centrifuge Rotofix 32 A (Hettich Zentrifugen, Tuttlingen, Germany) was used.

4.2 METHODS

4.2.1 Analytical Methodology for Eugenol Quantification

Eugenol was determined, detected and quantified through UV-Vis Spectrometry method.

Stock and Standards Preparation

A stock solution of 1648.0 ± 166.7 mg/L of eugenol was prepared in ultrapure water, by precisely weighting 82.40 ± 0.01 mg of eugenol from the analytical eugenol solution into a 50.00 ± 0.06 mL volumetric flask, using an analytical balance. In addition, a stock solution of 345.0 ± 100.0 mg/L of eugenol was prepared in octanol, by precisely weighting 34.50 ± 0.01 mg of eugenol from the standard solution into a 100.0 ± 0.1 mL volumetric flask, using the same equipment. Both stock solutions were sealed with parafilm, wrapped in aluminium foil to protect from light, and stored for 24 hours at 4 °C in the fridge in order to stabilize. For UV-Vis Spectrometry, three sets of working standard solutions of eugenol in a range of pre-defined concentrations were prepared in 10.000 ± 0.025 mL volumetric flasks by dilution of the stock solution in ultrapure water (Table 7). Moreover, another set of working standard solutions of eugenol also in a range of pre-defined concentrations, in 10.000 ± 0.025 mL volumetric flasks, was prepared by dilution of the stock solution of octanol. (Table 7).

Table 7 - Summary of the concentrations used for the preparation of the standard solutions of eugenol in all the four mediums investigated

Medium	Concentration Range (mg/L)
Ultra-pure Water	4.0 - 18.0
Basic Medium	2.0 - 12.0
Acid Medium	2.0 - 30.0
Octanol	0.5 - 20.0

All of these standard solutions were stored for 2 hours in fridge at 4 °C after being sealed with parafilm and protected from the light with aluminium foil, before analysis in the UV-Vis Spectrophotometer. All the measurements were performed using 10 µL, 50 µL, 100 µL, and 500 µL Hamilton Syringes (NV, USA). Regarding the acid and the basic medium, these solutions were prepared by adding HCl or $NaHCO_3$ into two different flasks of 150 mL within ultra-pure water in order to obtain a medium with pH 2 (acid) and other with pH 9 (basic), respectively.

Analytical Conditions and Instrumentation

All the spectrometry analyses were performed using three different types of UV-Vis Spectrophotometers. To determine the best detection wavelength for analysis, in which the maximum absorption of eugenol occurred, two intermediate solution of eugenol in ultra-pure water and in octanol, both with a concentration of 10 mg/L, were scanned between wavelengths of 190 to 900 nm and both the maximum absorption spectra in the two mediums were obtained (A9 in Appendix Section). Considering the maximum absorption wavelength from the spectra,

detection and quantification of eugenol was performed at 279 nm and at 236 nm, in all spectrometry analyses using ultra-pure water and octanol, respectively. For all the absorbance measurements, UV high precision quartz cells with 10 mm of light path were used, as well as the SPECTRA MANAGER Software. Five standards of eugenol were analysed for ultra-pure water and for ultra-pure water with $NaHCO_3$ (basic medium). In addition, fifteen working standards solutions of eugenol were analysed for ultra-pure water with HCl (acid medium), and twelve external standards of eugenol were analysed for octanol, as described in Table 7. Therefore, it was necessary to obtain a linear relation through the realization of four calibration curves for the four different mediums used.

Analytical Method Validation

The validation of an analytical method consists in obtaining a set of statistical parameters that characterize the respective method allowing to prove that the method is valid, meaning that it serves the purpose for which it was intended to, namely to control the variables that affect the results. Therefore, UV-Vis Spectrophotometry method was validated in order to: (1) assure that the eugenol detection was correctly performed; (2) demonstrate that this method was suitable for the reliability of the results regard the quantitative determination of eugenol; and to (3) be used as the analytical method of the release profiles of eugenol from the microparticles.

The validation performance parameters considered were the quantification parameters (linearity, sensitivity and limits of detection and quantification) and the reliability parameters (repeatability and intermediate precision). Regarding the quantification parameters, linearity is considered the ability of a method to demonstrate that the results obtained are directly proportional to the concentration of the compound in the sample within a specific range. Consequently, the results of the standard solutions analysed (for the four mediums) were processed statistically to determine the calibration curves equations and the correlation coefficients (R^2), using the Microsoft Excel software. Therefore, the linearity of the method was represented by the following calibration curve (Equation 2).

$$y = (a \pm ts_a)x + (b \pm ts_b) \quad \text{(Equation 2)}$$

Where a represents the regression slope, b represents the intercept of the regression and both ts_a and ts_b represent the standard deviation of the respective variables.

Moreover, for the calibration curves validation, the linearity of the process should be evaluated assuming that the following conditions and parameters are met:

- ✓ analysis of at least 5 standard concentrations
- ✓ linearity range in a factor superior to 10

- ✓ correlation coefficient (R^2) ≥ 0,99
- ✓ relative standard deviation of the slope $\frac{s_a}{a} \leq 5\%$
- ✓ calibration curve straight through the origin ($b\text{-}ts_b < 0 < b+ts_b$)

Furthermore, another parameter that characterizes an analytical method is the sensitivity defined as the ratio between the instrumental response and the concentration, i.e., the slope of the calibration curve. Therefore, all the calibration curves were constructed plotting the total values of absorption in the vertical axis and the eugenol standards concentrations in the horizontal axis. In addition, the limit of detection (LOD) is the minimum concentration of the sample from which it is possible to deduce and detect the presence of the compound, with a certain statistical certainty. The limit of quantification (LOQ) is the smallest concentration of the sample that can be measured and determined with an acceptable degree of accuracy and precision. Therefore, LOD and LOQ were determined based on the standard deviations of the responses and on the slope of the calibration curve using Equations 3 and 4.

$$LOD = 3 \times \frac{s_b}{a} \quad \text{(Equation 3)}$$

$$LOQ = 10 \times \frac{s_b}{a} \quad \text{(Equation 4)}$$

Regarding the reliability parameters, the precision of a method is defined as the degree of proximity between the results obtained in all the measurements referring to the same sample. It could be expressed by repeatability and intermediate precision, depending on the type of determinations performed. For repeatability, an intra-day measure, six analyses at three levels of concentrations (low, intermediate, high) were carried-out on the same day. For intermediate precision, an inter-day measure, three analyses at three levels of concentrations (low, intermediate, high) were carried-out in three non-consecutive days, being possible to vary at least one of the experimental conditions (measurement day). Additionally, for the ultra-pure medium, 4.0, 8.0, and 18.0 mg/L were the external standards concentrations used. The low, the intermediate and the high concentrations were, respectively, 2.0, 8.0, and 12.0 mg/L, for the basic medium (pH 9) and, 2.0, 14.0, and 30.0 mg/L, for the acid medium (pH 2). Lastly, for octanol medium, 0.5, 10.0, and 20.0 mg/L were the same working standards concentrations considered for this purpose. These parameters were all evaluated by the mean (M), standard deviation (s) and relative standard deviation (RSD).

4.2.2 Preparation of eugenol microparticles by Double Emulsion Solvent Evaporation Technique

In the present project, four sets of microparticles experiments where carried-out in triplicate (Table 8), varying different formulation parameters during the three phases of the

method, namely: the volume of W_1 phase used ranged between 1.0 mL and 2.0 mL; the organic solvent used in the organic phase varied from DCM/MeOH mixture to just only DCM; and the concentration of the polymer used as surfactant (PVA) in the W_2 phase ranged between 2% (w/w) and 1% (w/w).

Table 8 - Summary of the four formulations of eugenol microparticles performed for this project

Phase	Formulation Parameter	F1 [a]	F2 [a]	F3 [a]	F4 [a]
Internal Aqueous Phase (W_1)	**Volume of W_1 phase (mL)**	1.0	1.0	2.0	1.0
Oil Phase	**Solvent used**	DCM/MeOH (1/1, v/v)	DCM/MeOH (1/1, v/v)	DCM/MeOH (1/1, v/v)	DCM
External Aqueous Phase (W_2)	**PVA concentration (%) (w/w)**	2	1	1	1

PVA - Polyvinyl alcohol; DCM - Dichloromethane; MeOH - Methanol; [a] - Mean of 3 experiments; F - Formulation.

$W_1/O/W_2$ Phases

Firstly, using the ultra-pure water stock solution already prepared (1648.0 ± 166.7 mg/L), a standard solution of the active ingredient with a concentration of eugenol of 50.6 mg/L was prepared on a volumetric flask of 10.000 ± 0.025 mL. This value of concentration was obtained through the calibration curve of eugenol in ultra-pure water and corresponds to a maximum absorbance of 0.9. The volumetric flask was filled up with ultrapure water to the height of the meniscus using a pipette, and the sample was kept in the fridge at 4 ºC for 2 hours, for further stabilization. This final solution was named "Internal Aqueous Phase – W_1", and the volume used was dependent on the intended formulation.

Afterwards, 300.00 ± 0.01 mg of the encapsulating agent (ethyl cellulose), with a concentration of 30 mg/mL, were weighted on a calibrated analytical balance using a spatula to an ambar flask of 25 mL. A mixture of solvents was prepared using 5 mL of dichloromethane and 5 mL of methanol (or was only used 10 mL of dichloromethane alone depending on the intended formulation), being this solution added to the previous polymer flask using a micropipette. All the procedure was made on top of aluminium foil to avoid cross-contamination. The sample was agitated in the vortex shaker for 1 minute and then it was taken to an ultrasound bath between 15 to 60 minutes, until the dissolution of the polymer was done. After that, if necessary, the sample was agitated in the vortex shaker for an extra minute to assure the complete dissolution of the polymer. This solution was named "Oil or Organic Phase – O".

Moreover, a solution of PVA 2% (w/w) was prepared by weighing 2.00000 ± 0.00001 g of the polymer to a flask of 100 mL, using a spatula and a calibrated analytical balance, as well as by weighing 98.00000 ± 0.00001 g of ultra-pure water to a flask of 150 mL. Afterwards, the flask with ultra-pure water was put inside a pan in an AREX Digital stirring plate when the temperature reached the 120 ºC, while the magnetically agitation was set to 950 rpm using

simple magnetic bars. The polymer was rapidly added to the water and, after approximately 30 min, the complete dissolution of the polymer was already done, and all the equipment was turn off. The polymer rested for 24 hours on the top of the counter, covered with aluminium foil to avoid contamination. In the day after, the magnet was removed, and ultra-pure water was added to the solution to compensate the losses of water by evaporation in the night since it leads to an unintended and unmeasurable increase in concentration. Furthermore, the filtration of the polymer solution was made in order to remove all the possible residues left. Additionally, using the exactly same procedure, a solution of PVA 1% (w/w) was also prepared, weighing 1.00000 ± 0.00001 g of the polymer to a flask of 100 mL, as well as by weighing 99.00000 ± 0.00001 g of ultra-pure water to a flask of 150 mL. These solutions were named "External or Continuous Aqueous Phase – W_2", and the concentration used was dependent on the intended formulation.

Microparticles formulation

After the formation of the previous phases, 1.0 mL (or 2.0 mL depending on the intended formulation) of the internal aqueous phase was added to the organic phase. Then, the mixture obtained was agitated in the vortex shaker for 3 minutes. Subsequently, as soon as the first emulsion was prepared, the solution obtained was added to the 150 mL flask containing the PVA solution and was homogenised in a high-performance homogenizer for 5 minutes at 3 000 rpm, in order to obtain the microparticles. After every usage, this equipment was cleaned with a dichloromethane special solution for washing purposes. At the next step, the final mixture was left in the fume hood agitating during 3 hours using simple magnetic bars under constant stirring (650 rpm), in order to allow the hardening of the microparticles, as the solvent evaporated. This procedure was done inside a closed fume hood with air currents in order to promote a greater and faster air exhaustion. Later, the sample was washed with 500 mL of distilled water and filtered using a Vacuum Filtration System and a G4 filter to remove possible residues and excesses of PVA. Furthermore, the powder obtained was removed directly from the filter to a 25 mL ambar flask using a spatula. Lastly, these microparticles were kept in the freezer for 24 hours at -20 ºC, and then they were lyophilized for 12 hours.

4.2.3 Characterization parameters of Eugenol Microparticles

Three microparticles characterization parameters were evaluated.

Product Yield

The Product Yield (%) was expressed as the ratio of the mass of powder obtained after the lyophilisation process (microparticles dried) and the mass of the polymer and the mass of eugenol used to prepare the dispersive phase (Equation 5).

$$Product\ yield\ (\%) = \frac{Weigh\ of\ MP\ dried}{Weigh\ of\ polymer + Weight\ of\ \ eugenol\ oil} \times 100 \quad \text{(Equation 5)}$$

Encapsulation Efficiency

The Encapsulation Efficiency (EE) (%) was evaluated considering the amount of eugenol oil associated with the totality of particles. It was calculated according to the ratio between the difference of the amount of residual eugenol oil free in the solution and the total amount of eugenol oil used to prepare the particles, and the same value of the total amount of eugenol oil used to obtain the final particles (Equation 6). This parameter was obtained through the absorbance measurement on UV-Vis spectrometer of the supernatant obtained after 2.5 mL of the microparticles solution has been centrifuged and filtrated using TERUMO syringes (Tokyo, Japan) with a 0.2 μm membrane filter to ensure that PVA residues were retained in the filter.

$$Encapsulation\ Efficiency\ \ (\%) = \frac{Total\ amount\ of\ eugenol\ oil - Free\ amount\ of\ eugenol\ oil}{Total\ amount\ of\ eugenol\ oil} \times 100 \quad \text{(Equation 6)}$$

Water Content

The Water Content (%) in microcapsules was determined by the difference between the mass of powder obtained before the lyophilisation process (microparticles to be dried) and the mass of powder obtained after the lyophilisation process (microparticles dried). To obtain a result expressed by percentage, the ratio between the previous difference and the mass of powder obtained before the lyophilisation process (microparticles to be dried), was done (Equation 7).

$$Water\ Content\ \ (\%) = \frac{Weigh\ of\ MP\ to\ be\ dried - Weigh\ of\ MP\ dried}{Weigh\ of\ MP\ to\ be\ dried} \times 100 \quad \text{(Equation 7)}$$

4.2.4 Particle Morphology and Particle Size Distribution

Using the Scanning Electron Microscopy technique was possible to analyse some parameters of the morphology of the microparticles obtained, such as: sphericity, presence of agglomerates, superficial smoothness, and superficial porosity. Three different levels of magnification were used: low (320x), medium (1000x) and high (2000x).

The particle size distribution, the size of the microparticles and the dispersity grade, were analysed by Laser Granulometry technique by a Particle Size Distribution Analyser named Coulter Counter-LS 230. The size distribution (polydispersity degree) was measured in terms of SPAN value (polydispersity degree) according to Equation 8.

$$SPAN = \frac{D_{90} - D_{10}}{D_{50}}$$ (Equation 8)

Where D_{90} is the diameter in which 90% of the particles are smaller or equal in size compared to that value; D_{10} is the diameter in which 10% of the particles are smaller or equal in size compared to that value; and D_{50} is the median diameter where half of the particles have a smaller size and half of them have a larger size compared to that value.

4.2.5 Sustained Release studies

The study of eugenol release profiles from the obtained microparticles was carried-out in several different mediums: ultra-pure water (pH 5.6), octanol, ultra-pure water with HCl (pH 2) and ultra-pure water with $NaHCO_3$ (pH 9). Furthermore, suitable vehicles systems for skin delivery applications in hydrophilic or lipophilic cosmetic formulations using ultra-pure water and octanol, respectively, as the mediums to incorporate the microparticles for topical usages; as well as suitable vehicles for internal body delivery in food products using acid and basic medium to correctly simulate the gastric and the intestinal fluids, were respectively recreated. All the analyses were performed at room temperature. Approximately 10 mg of the lyophilized microparticles were suspended in 1.5 mL of each medium and magnetically stirred at a constant and low agitation speed (using simple magnetic bars). All of the samples were analysed at defined time intervals for the first hour, and then the absorbance values were collected every hour during 24 hours. The release analysis were accomplished by evaluating the amount of eugenol released from the particles over this specified time using a UV-Vis spectrometer.

4.2.6 Quality Assurance and Control

Eugenol is an essential oil very sensitive to light, heat and oxygen. A stock solution of eugenol in ultra-pure water and other in octanol were both preserved in the fridge (4 ºC) for 24 hours. Working standards solutions of eugenol were prepared and stored for 2 hours in the fridge (4 ºC). All of the solutions were sealed with parafilm and wrapped in aluminium foil to protected from light and avoid degradation, as well as to avoid the contamination of the reagents and products existing in the fridge. The ambar flasks containing the microparticles, after the filtration step, were also preserved using the parafilm and the aluminium foil and then stored in the freezer (-20 ºC) for further analysis.

4.2.7 Waste Treatment

The waste generated during the present work consisted of aqueous solutions containing eugenol, ethyl cellulose, methanol, dichloromethane, PVA, ultrapure water, HCl and $NaHCO_3$. All

of these residues were collected in closed containers, properly labelled for further treatment by the Environmental Management System of FEUP – EcoFEUP. As an exception, all the octanol waste was put inside a different compartment for later recovery.

5 RESULTS AND DISCUSSION

The results obtained are described: the Analytical Method Validation (5.1) was analyse; in Subchapter 5.2 the results of encapsulation efficiency, product yield, and water content are presented (Microparticles Characterization); in Subchapter 5.3 (Particle Morphology and Particle Size Distribution) the final particles were characterized regarding their morphology, size and size distribution; and the results of the release behaviour of the microparticles according to the mediums studied are analysed in Subchapter 5.4 (Sustained Release Study). The description of the processing conditions used through the different formulations studied can be seen in Table 8.

5.1 Analytical Method Validation

Considering the requirement to evaluate the percentage of eugenol released from the microparticles obtained during the sustained release studies, it was necessary to focus on analytical methods for eugenol quantitative determination by UV-Vis Spectrometry. The absorption spectra of eugenol in UPW and in OCT was obtained between wavelengths ranging from 190 to 900 nm in order to determine the wavelength of maximum absorption: 279 nm in ultra-pure water and 236 nm in octanol (A9 in Appendix Section). To obtain a linear relation, four sets of working standard solutions of eugenol in a range of pre-defined concentrations were analysed in four different mediums (Table 9). These results allowed the construction of four calibration curves for the sustained release studies in the four investigated mediums (Figure 7), where Abs represents the absorbance and C represents the concentration of the solutions.

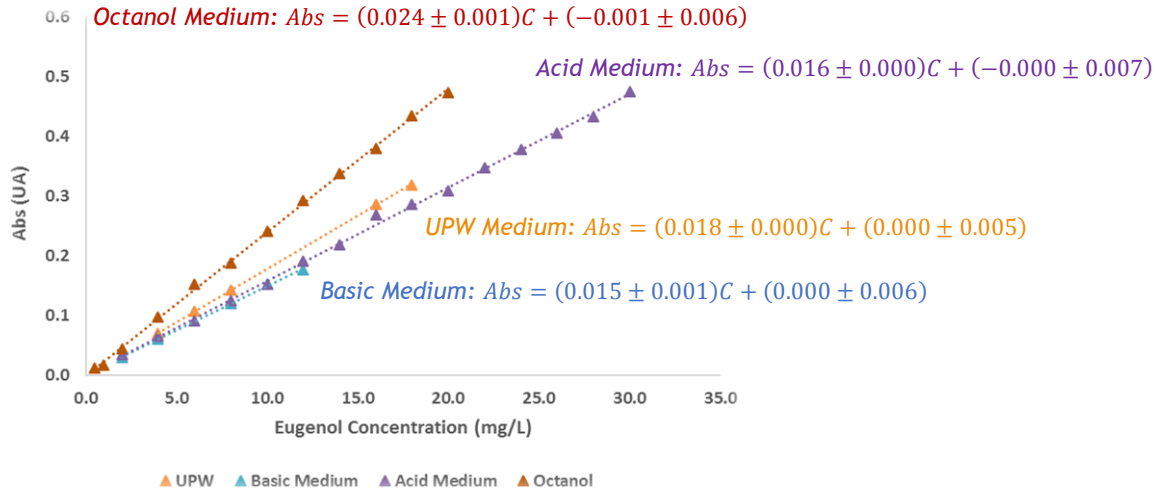


Figure 7 - Calibration curves of eugenol for the validation of the UV-Vis Spectrometry method in UPW, Basic Medium (pH 9), Acid Medium (pH 2) and OCT

The results of the linearity parameters of the UV-Vis Spectrometry method for eugenol quantification in the four studied mediums are presented in Table 9.

Table 9 - Linearity parameters for the validation of the UV-Vis Spectrometry calibration curves for eugenol quantification in UPW, Basic Medium (pH 9), Acid Medium (pH 2), and OCT

Parameters	Medium for release			
	UPW	Basic Medium (pH 9)	Acid Medium (pH 2)	OCT
Number of standards concentrations	5	5	15	12
Linearity Range (mg/L)	4.0 - 18.0	2.0 - 12.0	2.0 - 30.0	0.5 - 20.0
Correlation coefficient (R^2)	0.999	0.999	0.998	0.999
Slope error (%) (S_a/a)	0.729	1.930	1.166	0.933
Intercept confidence interval ($b-t_{sb}<0<b+t_{sb}$)	-0.001<0<0.002	-0.002<0<0.002	-0.004<0<0.003	-0.003<0<0.002
LOD (mg/L)	0.258	0.351	0.636	0.317
LOQ (mg/L)	0.860	1.171	2.121	1.058

UPW - Ultra-pure water; OCT - Octanol; LOD - Limit of Detection; LOQ - Limit of Quantification.

The calibration curves obtained were linear for all the four mediums as well as in the studied concentration ranges. Therefore, the results obtained about the linearity of the calibration curves were, generally, very satisfactory because the linearity parameters required for the method validation, already described, were all achieved. Additionally, the values obtained for the correlation coefficient (R^2) were near the unit value (≥ 0.99). The LOD results were lower than the lowest standards concentration used for the calibration curves allowing us to conclude that it is possible to detect the presence of the compound in the concentrations conditions in which the analysis was done. The LOQ values were also lower than all the concentration values analysed in the sustained release study, meaning that the concentrations used in the samples were sufficient to be measured and determined with a satisfactory degree of accuracy and precision.

Regarding the reliability parameters, the results obtained in all the four mediums, expressed by M, s and RSD, are presented in A1-A8 (Appendix Section). The data showed that there was no significant response variations, since the overall repeatability and intermediate precision results for the four mediums showed relative standard deviation values lower than 5% (highly precise method). Nevertheless, it was possible to detect some exceptions in these results, mainly due to possible instrumental or operator errors, as well as due to the degradation of the solutions. Therefore, it is possible to conclude that the overall precision results, in all the measurements referring to the same samples, were highly precise since the RSD values were small. That leads towards the conclusion that the data obtained are strongly grouped around the mean, being the results very closed to each other.

5.2 Microparticles Characterization

Some characterization parameters of the microparticles were calculated. The results obtained for the EE, PY, and WC are described in Table 10 and in A10 in Appendix Section.

Table 10 -Microparticles characterization parameters obtained for the four formulations

	PVA 2%, DCM/MeOH, 1.0 mL		PVA 1%, DCM/MeOH, 1.0 mL		PVA 1%, DCM/MeOH, 2.0 mL		PVA 1%, DCM, 1.0 mL	
	F1		F2		F3		F4	
	Mean [a] (M ± s) (%)	RSD (%)	Mean [a] (M ± s) (%)	RSD (%)	Mean [a] (M ± s) (%)	RSD (%)	Mean [a] (M ± s) (%)	RSD (%)
Encapsulation Efficiency	84.0 ± 3.5	4.2	76.4 ± 21.6	28.3	93.3 ± 0.2	0.2	92.9 ± 0.3	0.4
Product Yield	80.7 ± 1.2	1.5	82.4 ± 2.6	3.2	79.2 ± 1.6	2.0	77.5 ± 6.1	7.9
Water Content	54.3 ± 10.1	18.6	56.5 ± 3.9	6.9	48.1 ± 7.2	14.9	87.5 ± 0.6	0.6

M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 experiments; F - Formulation.

Encapsulation Efficiency

The encapsulation efficiency of eugenol (amount of eugenol effectively encapsulated within the particles) was 84.0 ± 3.5% for F1, 76.4 ± 21.6% for F2, 93.3 ± 0.2% for F3, and 92.9 ± 0.3% for F4, respectively. The highest ratio was obtained in F3 and the lowest ratio was obtained for F2. The data obtained suggest that the volume of internal aqueous phase used could be considered the predominant factor that influences the EE of the process since this volume is directly associated to the amount of eugenol available to microencapsulate. Therefore, in F3, a OWR ratio of 5 was used (10 mL of solvent and 2.0 mL of internal aqueous phase), while in F4 a OWR ratio of 10 was used (10 mL of solvent and 1.0 mL of internal aqueous phase), suggesting that EE values increased with the decrease of the OWR from 10 to 5, i.e., as the volume of the internal aqueous phase increase from 1.0 to 2.0 mL, allowing to conclude that more eugenol is available to encapsulate is associated to higher encapsulation efficiency. These results are in line with Lamprecht et al. (2000), whom suggested that a higher concentration (i.e. smaller volume) in the internal aqueous phase would lead to a decrease in the encapsulation efficiency of particles performed by double emulsion pressure homogenization technique, as it was also reported in the present project. However, the results obtained by Zambaux et al. (1998) showed the contrary, i.e., that an increase in the volume of the internal aqueous phase, in the double emulsion method, would decrease the EE of the active ingredient, which was not observed in the present study. However, between F3 and F4, the EE results obtained were very similar between each other (93.3 ± 0.2% and 92.9 ± 0.3%, respectively).

Furthermore, an increase in EE between the first three formulations and F4 might be explained by the usage of just DCM as a solvent in F4, leading to a slower transfer of dispersive phase to continuous phase, allowing a better encapsulation efficiency.

A study that encapsulated eugenol in β-cyclodextrin (β-CD) particles by molecular inclusion method obtained 91.7% of EE after freeze-drying (Seo et al., 2010). In addition, Choi et al. (2009) also investigated the encapsulation of eugenol by molecular inclusion method with β-CD and 2-HP-β-CD, as well as by an emulsion–diffusion method with poly(caprolactone) (PCL). On that study for EE results of PCL, β-CD and 2-HP-β-CD eugenol inclusion complexes, there were found values such as 100%, 90.9% and 89.1%, respectively. Therefore, this study revealed that the emulsion–diffusion method was more efficient and effective than the molecular inclusion method for eugenol encapsulation. This may be due to their complete wrapping of eugenol by PCL layer suggesting that the EE results would be higher in the first technique (Choi, et al., 2009). Moreover, according to Gomes et al. (2011), spherical PLGA nanoparticles with entrapped eugenol were also produced by emulsion evaporation method using PVA as a surfactant, allowing them to obtain 98% of EE for eugenol. Likewise, in another study by Woranuch and Yoksan (2013), eugenol was encapsulated into chitosan nanoparticles via an emulsion–ionic gelation crosslinking method, i.e., first there was the formation of an oil-in-water emulsion and then the ionic gelation of emulsion droplets. The obtained particles presented an EE in the ranges of 1.1–20.2%, which were very low values compared to the ones obtained in this project by double emulsion solvent evaporation technique.

In the present study, the EE ranged between 76.4 ± 21.6% to 93.3 ± 0.2%. These values are lower than the ones obtained in emulsion evaporation (98%) or emulsion diffusion techniques (100%) previously reported for eugenol (Choi et al., 2009; Gomes et al., 2011). However, the EE obtained in F3 and in F4 (93.3 ± 0.2% and 92.9 ± 0.3%) were higher when comparing to EE of the β-CD eugenol particles (90.9% and 89.1%) (Choi et al., 2009). In addition, the EE obtained in all the four mediums was higher compared to the EE results reported by Woranuch and Yoksan (2013). Likewise, other study in which eugenol was effectively encapsulated with chitosan polymer by spray-drying technique (Pereira, 2007), the EE ranged between 50-82% being again lower than the main data obtained in our study. Therefore, all of these results allowed me to suggest that the double emulsion evaporation technique used in the present study was a suitable choice for the encapsulation method of eugenol molecule. Despite that, according to Meng et al. (2004) and Cohen-Sela et al., (2009), using a W/O/W double emulsion technique with diffusion of the solvent was reported as a method with better encapsulation efficiencies compared to the solvent evaporation technique (90 vs 30% and 87 vs 55%, respectively). The same approach was also used by Meng et al. (2003) which obtained values above 94% for the microparticles encapsulation efficiency.

Product Yield

The product yield (quantity of powder recovered in relation to the amount of raw materials used) was 80.7 ± 1.2% for F1, 82.4 ± 2.6% for F2, 79.2 ± 1.6% for F3, and 77.5 ± 6.1% for F4, respectively. The highest value was obtained for F2, while the lowest value was obtained for F4. Being the organic solvent used, the only difference between those formulations, it is possible to suggest that the organic solvent used for the organic phase could be consider as a parameter that affects the product yield of the process, being achieved a higher PY when a mixture of DCM/MeOH is used. Therefore, when a high water-solubility organic solvent like methanol is used as co-solvent in the mixture with high immiscible solvents as DCM (formulations 1, 2 and 3), the mass-transfer between the dispersive and the continuous phases is faster, leading to a faster precipitation of the polymer and, therefore, to higher product yields. This could be the basis of explanation for the lower PY value obtained in F4 (77.5 ± 6.1%) were only DCM was used as organic solvent. However, in general, all the results among the totality of the experiments were similar, confirming the reproducibility of the method as it was expected.

Gong et al. (2016) encapsulated eugenol by inclusion method into β-CD particles, in which the βCD–eugenol inclusion complex was recovered as a pale yellow solid with a yield bigger than 90%. In fact, the inclusion of essential oils into β–CD allows that several chemical and physical properties of the encapsulated molecules are optimized, increasing their solubility and stability, as well as masking certain undesirable physiological effects (e.g. odours) and reducing the volatility (Gong et al., 2016). Therefore, since at the best of our knowledge any other comparable data was found about PY parameter for eugenol, it might be possible to conclude that the increase in stability and the reduction of the volatility characteristics of the inclusion method, allowed the β–CD complex to achieve higher PY results compared to the ones obtained by double emulsion solvent evaporation technique used in the present project (77.5 ± 6.1% to 82.4 ± 2.6%).

Water Content

In this study were found values of 54.3 ± 10.1%, 56.5 ± 3.9%, 48.1 ± 7.2% and 87.5 ± 0.6% for the WC of the final eugenol microparticles in formulations 1, 2, 3 and 4, respectively. Whereas in the first three formulations this parameter is in a range between 48.1 ± 7.2% and 56.5 ± 3.9%, in F4, however, the WC result was unexpectedly high reaching 87.5 ± 0.6%. One possible explanation may lie on the solvent used. Indeed, the principal change in F4 was that we used DCM as a solvent, while in formulations 1, 2 and 3, it was used the DCM/MeOH mixture instead. Consequently, this difference may likely be explained due to the high immiscibility of DCM with water, since more water molecules may be retained in the bulk of microparticles (Birnbaum et al., 2000). Additionally, small alcohol molecules like methanol are easily dissolved

in water (hydrophilic compound) allowing the water to be able to form hydrogen bonds with the highly reactive hydroxyl group of methanol, ensuring a high miscibility between the inner aqueous phase and the outer aqueous phase and, consequently, justifying the lower WC values obtained for the first 3 formulations. According to Yeo and Park (2004), when a high water-solubility organic solvent like methanol is used as co-solvent (formulations 1, 2 and 3), there are relatively fast mass-transfer between the dispersive and the continuous phases leading to a faster precipitation of the polymer. Additionally, being this solvent a water-miscible solvent, it is expected that it facilitates the diffusion of water into the dispersive phase. Therefore, this could be the explanation why in F4, where DCM was used alone, a high percentage of water content was obtained and, therefore, the diffusion of water was not done correctly or effectively.

The residual WC of the final microparticles after the freeze-drying process was already investigated in order to analyse the thermal properties of the β-CD eugenol particles (Choi et al., 2009). Additionally, in a study conducted by Seo et al. (2010), the influence of parameters like humidity and storage temperature on the water content increment of βCD-eugenol powder were studied, due to the rapid dehydration of the complex.

5.3 Particle Morphology and Particle Size Distribution

The eugenol microparticles obtained in the present study were analysed by SEM for the respective particle morphology analysis (Figure 8, 9 and 10, for high (2000x), medium (1000x) and low (320x) magnifications, respectively). Moreover, these microparticles were also analysed by laser granulometry to determine mean size and particle size distribution.

Particle Morphology

In F3, a highly spherical, smooth and regular, almost without pores (the existing ones are truly small) microparticles population was obtained, also confirming the smaller mean size (128 ± 0.2 μm) and higher polydispersity degree (2.3 ± 0.0) obtained in this formulation, since the particles seems to have different sizes but in general, smaller ones (Figure 8). For F1, it is possible to confirm that the particles are bigger (132 ± 14.8 μm) and more similar between each other (SPAN F1; 1.9 ± 0.1), but showing some spherical and other non-spherical microparticles mixed (some quadrilateral), with more pores (Figure 9). On the opposite, for F2, although the microparticles have an irregular and not totally spherical morphology, it seems they have a smooth surface with larger pores. In F4, the final particles showed to be irregular and non-spherical in shape with a very rough and harsh surface, where indentations, wrinkles and several large pores appeared on the microparticles surface, giving them the typically appearance of a sponge, and others seemed crystals. Therefore, being the only difference between F4 and the

other 3 formulations the organic solvent used, it was expected that the higher solubility of DCM/MeOH mixture would lead to a longer evaporation time and, consequently, to smooth and non-porous surfaces. In the present study, that was observed (Figure 10), since in F4 where just DCM was used as solvent, a faster evaporation happened leading to atypical surface morphologies with rush surfaces and large pores.

Furthermore, it is possible to observe that also in F4, the agglomeration of the microparticles happened (Figure 9). Therefore, it was already reported that the aggregation present on particles could be likely due to natural clustering due to insufficient steric stabilization by the PVA, which is a non-ionic surfactant formed from alternating hydrophilic and hydrophobic segments (Gomes et al., 2011). This may explain why in F2, a tendency to obtain agglomerates was also observed in some of the microparticles, since in this formulation a smaller concentration of PVA (1%) was used in the external aqueous phase and, therefore, less PVA molecules were covering and protecting the interface of the microparticles. Generally, the distinct difference in the surface morphology of the microspheres could be explained by their different stability of primary emulsion, as reported by Meng et al. (2004). Another interesting conclusion which is possible to take from a different study, is that it is expected that a higher concentration of the core ingredient (smaller volume) leads to roughs and porous surfaces (Das and Rao, 2007), which was observed in the present study since in F3, where 2.0 mL of internal aqueous phase was used, the most smooth, regular and spherical particles where obtain (Figure 10). Moreover, it was confirmed a direct correlation between morphology parameter and encapsulation efficiency in the present study, since 93.3 $\pm$ 0.2% of EE was obtained in F3, exactly where in general the morphology of the particles had better quality (smooth and spherical surface without pores). The correlation between the microparticles morphology and the final accumulated release percentages after 24 hours is going to be further explained.

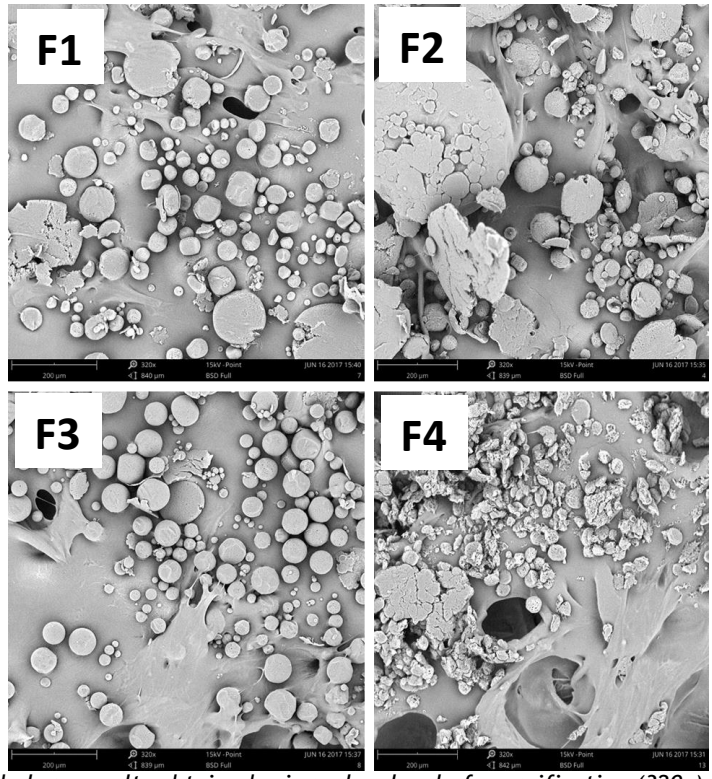


Figure 8 - Particle morphology results obtained using a low level of magnification (320x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right)

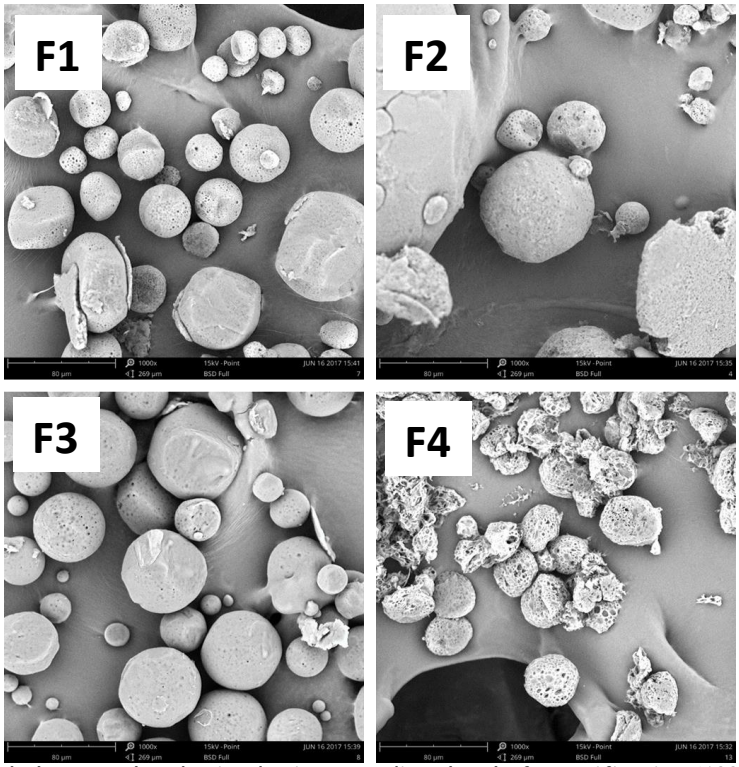


Figure 9 - Particle morphology results obtained using a medium level of magnification (1000x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right)

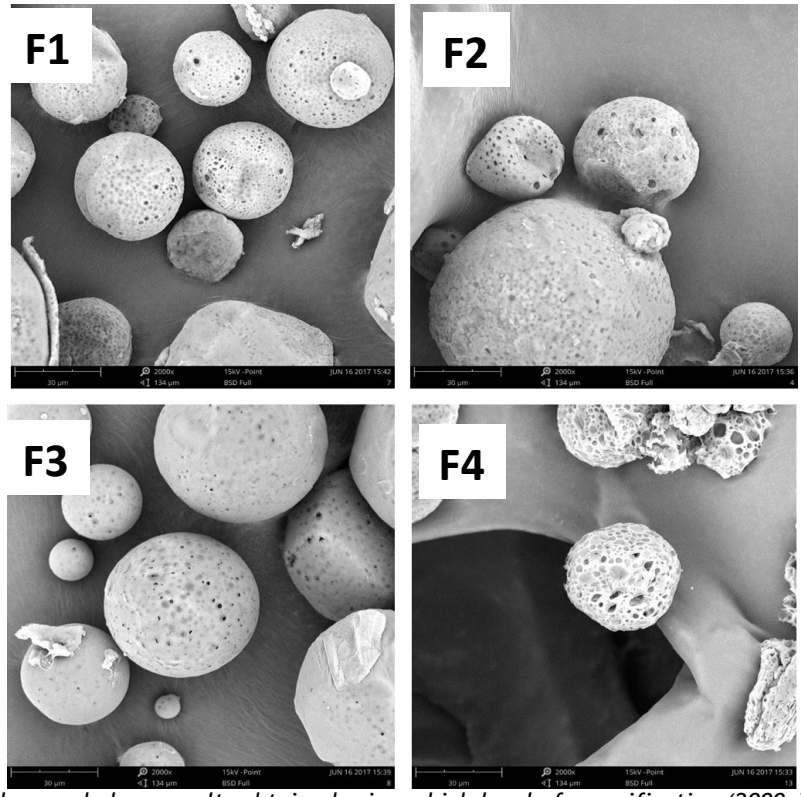


Figure 10 - Particle morphology results obtained using a high level of magnification (2000x) for F1 (top left), F2 (top right), F3 (bottom left) and F4 (bottom right)

Additionally, transmission electron microscopy (TEM) was already used as morphology analysis technique in eugenol studies (Seo et al., 2010; Woranuch and Yoksan, 2013). In addition, several authors have already investigated the morphology analysis of encapsulated eugenol by SEM technique, was it was performed in the present project. Therefore, according to Choi et al. (2009), the eugenol encapsulated by PCL presented a rounded shape and a smooth surface, while the eugenol inclusion complexes of β–CD presented rough and irregular forms, with some agglomeration of the particles, allowing us to suggested that the double emulsion solvent evaporation technique used in the present study was a positive choice in order to obtain microparticles with better morphologic characteristics. Additionally, Gomes et al. (2011) also encapsulate eugenol, obtaining particles which spherical shape and smooth surface but with a strong tendency to form clusters. Moreover, SEM images of EUG-β–CD complexes were also obtained by Piletti et al. (2017), where crystalline structure formed by crystals with different sizes and with irregular and harsh surfaces was observed, as well as Gong et al. (2016), whom showed a tendency to form agglomerates for βCD-EUG complexes, with a compact crystalline structure of it. Additionally, similar morphology results were obtained by double emulsion technique (Das and Rao, 2007; Lamprecht et al., 2000; Meng et al., 2004; Nihant et al., 1994; Prasertmanakit et al., 2009; Meng et al., 2003; Zambaux et al., 1998) encapsulating vitamins, drugs and proteins.

Particle Size Distribution

The mean particle size and the size distribution of the particles are considering important parameters regarding the production and development of suitable micron-size delivery systems for industrial applications, possibly affecting the microparticles characterization parameters as well as the release profile of the active compound. A lower polydispersity degree and smaller size of microparticles are normally preferred. The results of this sub-chapter are presented in Table 11, described with more detail in the Appendix Section (A11-A16).

Table 11 - Particle mean diameter and polydispersitivity degree results for the four formulations

	PVA 2%, DCM/MeOH, 1.0 mL		PVA 1%, DCM/MeOH, 1.0 mL		PVA 1%, DCM/MeOH, 2.0 mL		PVA 1%, DCM, 1.0 mL	
	F1		F2		F3		F4	
Parameters	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± s)**	**RSD (%)**
Mean (μm)	132 ± 14.8	11.2	196 ± 18.6	9.5	128 ± 0.2	0.1	850 ± 19.1	2.3
SPAN	1.9 ± 0.1	7.1	1.8 ± 0.1	6.2	2.3 ± 0.0	0.4	2.0 ± 0.1	3.3

s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions; SPAN - Polydispersitivity degree; F - Formulation.

The mean diameter values obtained in this study were: 132.0 ± 14.8 μm for F1; 196.0 ± 18.6 μm for F2; 128.0 ± 0.2 μm for F3, and 850.0 ± 19.1 μm for F4. Therefore, between F1 and F2, the particle size increased from 132 ± 14.8 μm to 196 ± 18.6 μm. The main difference between those formulations was the concentration of stabilizer (PVA) used, that was bigger (2%) in the F1 were the mean diameter was smaller (132 ± 14.8 μm). This result agrees with Zambaux et al. (1998) and Prasertmanakit et al. (2009), which suggested that an increase in surfactant concentration in the external aqueous phase would result in size reduction, due to the expected increase on frequency of collision of droplets leading to lower particles sizes (Tiwari and Prerana, 2011), which was reported in the present study.

Further, between F2 and F3, the particle size decreased from 196 ± 18.6 μm to 128.0 ± 0.2 μm, and this could be explain for the fact that a higher volume (2.0 mL) in internal aqueous phase was investigated. According to Zambaux et al. (1998), it was expected that a higher internal aqueous phase volume contribute to a smaller particle size, due to the fact that should occurs a greater partionating of the internal aqueous phase in the organic phase, like it was also obtained in the present project.

Lastly, the mean particle size highly increased from F3 to F4 from 128 ± 0.2 μm to 850 ± 19.1 μm and that is in line with Zambaux et al., (1998) suggestions because the volume of the internal aqueous phase was rearranged again to 1.0 mL leading to an increase in mean size. In addition, it is also known that the type of solvent used could affect the particle size as well (Iqbal et al., 2015). Therefore, this may also be the basis of explanation for the same increase of particles sizes, since in F4 only DCM was used as organic solvent while on the other three formulations a mixture of DCM and MeOH was chosen. For instance, methanol is water-miscible

while dichloromethane is water-immiscible solvent. Therefore, the mixture of both the solvents will increase the water solubility of the solution. According to Jelvehgari and Montazam (2012), this may result in an extraction of the solvent by the external phase and an interfacial turbulence may occur between the organic polymer phase and the external aqueous phase leading to the formation of smaller particles. Therefore, when comparing formulation 1, 2 and 3, where a mixture of DCM and MeOH was used, and F4, where just DCM was used, those suggestions are confirmed once the obtained final particles were bigger when just DCM was used (F4; 850 $\pm$ 19.1 μm). Moreover, it should be noted that the larger size of particles observed in F4 compared to the ones obtained in the other formulations, might also be a result of aggregation of the microparticles.

Several parameters that may influence particle size and size distribution of the microparticles, were already discussed previously (Birnbaum et al., 2000; Iqbal et al., 2015; Jelvehgari and Montazam, 2012; Lamprecht et al., 2000; Leimann et al., 2009; Stella et al., 2017; Zambaux et al., 1998). According to Gong et al., (2016), was possible to obtained micron-sized particles with 4.2 μm by the βCD-EUG inclusion complex produced. However, it is possible to conclude that almost all the particle sizes results reported for encapsulated eugenol were obtained in a nanometre scale (Choi et al., 2009; Gomes et al., 2011; Hu et al., 2016; Majeed et al., 2016; Seo et al., 2010; Sharif et al., 2016; Woranuch and Yoksan, 2013).

Regarding the volumes distribution (A16 in Appendix Section) it is possible to conclude that some disturbances were found out, being the curves not normally distributed in the generality of the experiments, were some bi-modal and tri-modal profiles were observed. Indeed, significantly amounts of larger particles were obtained in F4 (confirmed during the practical analysis), which may be explained by aggregation effects leading to worst distributing curves. Furthermore, the results about the volume distribution of 10%, 50% and 90% allowed the determination of the SPAN value, i.e., the polydispersitivity degree of the microparticles, representing the distribution of particles sizes. In our study, the biggest value for SPAN was 2.3 $\pm$ 0.0 in F3 compared to the other formulations were the values were, in general, all similar between each other (1.9 $\pm$ 0.1 for F1; 1.8 $\pm$ 0.1 for F2; 2.0 $\pm$ 0.1 for F4). Therefore, it is possible to conclude that in F3 the particles presented different sizes among themselves (bigger polydispersitivity), while in the other formulations (formulations 1, 2 and 4) the particles presented a smaller polydispersitivity degree, showing more similar particle sizes between each other. Consequently, given the fact that the main difference in F3 was the internal aqueous phase volume, in comparison with the other formulations, it is possible to suggest that this parameter may be a main factor that influences the polydispersitivity degree of the microparticles obtained as already reported by Zambaux et al. (1998). Nevertheless, the results obtained by Zambaux et al. (1998) suggested that a bigger volume of internal phase could

eventually lead to a lower polydispersity degree that, on the opposite, was not observed in F3 of the present study, where using 2.0 mL of internal aqueous phase increased the SPAN to 2.3 ± 0.0. However, this was not entirely significant since all the SPAN values obtained were comparable (1.9 ± 0.1, 1.8 ± 0.1, 2.3 ± 0.0 and 2.0 ± 0.1 for formulations 1, 2, 3 and 4, respectively).

Furthermore, polydispersity degree lower than the ones obtained, were already reported by several authors: ranging between 0.1–0.5 (Hu et al., 2016); between 0-0.2 (Sharif et al., 2016); approximately 0.1 or 0.2 reported by Majeed et al. (2016); in a range of 0.2-0.4 according to Choi et al. (2009); and 0.3 for two studies (Gomes et al., 2011; Seo et al., 2010). Therefore, it was suggested that the main advantage of a monodisperse system is its ability to deliver a consistent amount of compound, when compared to a mixture of polydisperse particles (Gomes et al., 2011). In the present study, all the dispersity degrees results were very similar and in a range between 1.8 ± 0.1 and 2.3 ± 0.0, leading to the conclusion that all the formulations obtained were polydisperse systems, which is in line with the suggestions made Bitar et al. (2015) whom suggested that the particles prepared by emulsification processes have non-uniform (polydisperse) sizes. Therefore, the amount of surfactant played an important role in the protection of the microparticles during the hardening process, because it can avoid the coalescence and agglomeration of them as it can be seen in the surface morphology results obtained for F2, where PVA 1% was used and, therefore, less PVA molecules were covering and protecting the interface of the microparticles increasing the tendency to create agglomerates (Figure 8).

5.4 Sustained release studies

The sustained release study of eugenol was planned to simulate both topical and internal body delivery conditions, as well as to study the consequent microparticles behaviour over 24 hours. The study was performed in ultra-pure water (pH ≈ 5.6) and octanol in order to simulate hydrophilic and lipophilic cosmetic formulations for skin, respectively; as well as in ultra-pure water with HCl and $NaHCO_3$ in order to simulate delivery conditions not only in the gastric tract (pH ≈ 2), like in stomach, but also in the intestine (pH ≈ 9), especially in the duodenum where the release of the compound is expected and where its chemical digestion occurs. All the release analysis were accomplished by UV-Vis Spectrometry method, in a maximum absorption of 279 nm for ultra-pure water and 236 nm for octanol. Then, it was evaluated the amount of eugenol released from the particles in predetermined time intervals over 24 hours at room temperature.

An overall picture of the obtained eugenol release profiles for each individual formulation in the four studied mediums is presented in A17 (Appendix Section). Therefore, is possible to observe that the mediums were there was more and less release of eugenol were not

concordant among the four formulations. For instance, for formulations 1 and 3, the best final accumulated release percentage was obtained in the acid medium (66% and 91%, respectively), for F2 the best final accumulated release percentage was obtained in OCT (48%), and for F4 the best final accumulated release percentage was obtained in the basic medium (62%). Additionally, OCT medium was the medium with less eugenol release for F3 (43%) and 4 (39%), and the basic medium and UWP were the ones with worst release profiles for F1 (7%) and F2 (22%), respectively. Moreover, Figures 11, 12, 13 and 14 compare all eugenol release profiles from the final microparticles in the four different mediums: ultra-pure water, basic medium, acid medium and octanol, respectively. For instance, F3 was the one showing best final accumulated release percentage in UPW (77%), basic medium (68%) and acid medium (91%), while in OCT the best result was found out for F1 (50%). The particles obtained in formulation 3 were the ones with a smother surface and the ones which almost had no porous. Therefore, it was expected to obtain lower accumulated releases in this formulation but different results were obtained. Consequently, it may be possible to suggest that the eugenol release through superficial porous is not the controlled release mechanism of eugenol from EC microparticles. In addition, F1 showed the lowest final accumulated release percentages for UPW (22%) and basic medium (7%), possible due to a higher amount of PVA protecting the particles and preventing eugenol release (PVA 2% was used); whereas for acid medium and OCT, F2 (38%) and F4 (39%) showed the lowest final accumulated release percentages, respectively, possible due to the fact that high agglomeration of the microparticles was found out on both of these formulations, preventing the release.

According to Seo et al. (2010) the release of entrapped flavouring materials like eugenol was closely related with the absorption of water by its penetration in the surface of the wall of the particles, followed by formation of holes, pores or cracks that appear near the particles' surface of the particles allowing the subsequent release of the molecule. Indeed, it was already reported that eugenol could be quickly release due to the dissociation of CD complex by the increment of hydration at high humidity and storage temperatures and, in that case, high water absorption was the main explanation for eugenol release rate in this medium (Seo et al., 2010). This could be in line with the present study where the formulation with higher water content (WC F4; 87.5%) was the formulation with one of the best eugenol release results in UPW (F4 in UPW; 42%), probably also due to the highly porous surface.

When analysing the basic and the acid medium release profiles, it is possible to conclude that the release was much faster when HCl was present in the solution because ethyl cellulose polymer is expected to be more stable within a basic solution rather than an acid solution since, in the presence of an acid, hydrolysis of ethyl cellulose ether groups is expected to happened. Therefore, as an example for basic medium, in Figure 12, it is possible to observe

low final accumulated release percentages in a general range between 25-70% for 24 hours, with an exception in F1 where even lower final accumulated release percentage was obtained (7%) in the same period of time. Moreover, the use of acid was already reported previously. According to Woranuch and Yoksan (2013), nanoparticles were digested in hydrochloric acid solution to break up the nanoparticles and allow the release of the encapsulated eugenol. In the present study, a release range between 40-90% was achieved in the acid medium and F3 was the one where a faster release profile was obtained (≈ 90% in 24 hours).

When analysing the UPW and OCT medium release profiles, eugenol microparticles showed a faster release profile in OCT compared to the release in UPW. In fact, a final accumulated release percentage of eugenol between 10-40% approximately, after 24 hours, was released in water compared to a range between 40-50% released in octanol during the same time. This difference among release behaviour in water and octanol is explained by the fact that ethyl cellulose is highly soluble in octanol and high insoluble in water. Therefore, when the polymer is in contact with octanol not only there is absorption of it (swelling capacity) but also erosion of the wall material allowing the disintegration of the microparticles and, consequently, the creation of pores allowing a faster release of the active ingredient. In the case of ultra-pure water, this is capable of establish hydrogen bonds with ethyl cellulose hydroxyl groups also creating surface pores in the polymer allowing the eugenol release. However, the interaction between water and ethyl cellulose is not enough for better final accumulated release percentages of eugenol, like the ones observed in octanol, since the degree of ethylation of the polymer is 47%, meaning that just approximately half of the ethyl cellulose can establish hydrogen bonds with water. The only exception found was reported for F3, in which it was released almost 80% of eugenol in water in 24 hours. This could be explained by the fact that a higher volume of internal aqueous phase was added, because that was the only one change made in this formulation compared to the others. Further, although in a very different study, Kayaci et al. (2013) reported a similar event, where a higher amount of released eugenol was obtained for PVA/EUG/α-CD compared to PVA/EUG because the eugenol content presented in the PVA/EUG/α-CD nanofibers was more than in PVA/EUG nanofibers, allowing the elution of surface-associated molecules by creating channels that subsequently also allow the elution of the active ingredient. As far as is known, there is no comparable data in the literature. Nonetheless, as F1 was the first study and no special condition was tested (control study), it is reasonable one suggest that all the other formulations in which at least one parameter was changed, influence the release profile of eugenol.

Yeo and Park (2004) suggested that a higher PVA concentration would result in higher viscosity of the continuous phase and smaller particle size, preventing migration of the internal aqueous phase towards the continuous phase, and consequently allowing a decreased in the

initial burst, which was confirmed in our study in the majority of the outcomes. Indeed, F1 was the formulation where PVA 2% was used and in general there was not a significant burst phase (A17 in Appendix Section) in the release profiles obtained. Additionally, Eltayeb et al. (2015) suggested that the release profiles could be influenced by the polymer properties (molecular weight and concentration), the nature of the core material, as well as the particle size distribution. Therefore, it was concluded that smaller particles normally tend to obtain higher release rates due to the higher surface area to volume ratio, allowing a rapid and better penetration by the external medium. Majeed et al. (2016) also reported a considerable increase in release percentage of eugenol with decreasing droplet diameter. Therefore, those suggestions were confirmed in our study. Indeed the F3 was the formulation with smaller particles (128 μm) and, consecutively, higher release profiles: 77% for UPW, 68% for basic medium and 91% for acid medium. The only exception was for octanol, where just 44% of eugenol was released.

In the present study, although some unusual results were observed, it can generally be summarized that the eugenol oil release profiles obtained for ultra-pure water, basic medium and acid medium presented: (a) a first initial burst release, possibly due to a rapid release of eugenol molecules which are adsorbed outside the microparticle, or to a rapid release of microencapsulated eugenol which is closed to the polymeric matrix surface being easily release; and (b) a slow increase together with a constant sustained release of the remaining eugenol with time, resulting from the erosion of the polymer wall and consequently the compound diffusion from inside particles towards the outside medium. An example of this type of profile it is the release profile of F2 and F3 in basic medium (Figure 12), and F1 in acid medium (Figure 14). It is possible to observe a clearly initial burst and, then, a consecutive sustained release until the 24 hours. In addition, one might conclude that in overall octanol release profiles, the eugenol microparticles exhibited a fast initial release (≈ 40% after 1 hour), along with a very similar sustained behaviour until the end of the time between the four formulations (plateau). Alternatively, for F3 in UPW (Figure 11), it is possible to observe a tri-phase type release profile: an initial burst release, followed by a uniform and slow second phase release within time (plateau) and, finally, a third rapid phase with an increase of eugenol release.

Therefore, according to Birnbaum et al. (2000), it was expected that microparticles prepared with a mixture of dichloromethane and methanol would give more constant release profiles than microparticles prepared using dichloromethane alone, which was confirmed in the present study where it is possible to conclude that more irregular release profiles were obtained for F4 (DCM alone as solvent). Furthermore, lyophilisation decreases the burst effect according to Tiwari and Prerana (2011), which may explain why not all the release profiles presented this initial phase since freeze-drying was the drying technique used in the present study. Furthermore, other sustained release experiments conducted in vitro at 37 ◦C and 100 rpm for 72

hours showed an initial burst followed by a slower rate of release of eugenol entrapped inside a PLGA matrix, obtaining 45% as the percentage of eugenol release for 5 h and 64% of the initial load of eugenol was released in the medium in 72 hours (Gomes et al., 2011). The rapid eugenol release reported by the previous author (20% under 0.5 hours) could be attributed to the diffusion of the encapsulated or adsorbed antimicrobial in the external surface of the nanoparticles, being the second part of the release (after 5 h) highly dependent of the time, which was related to the core material diffusivity inside the matrix system, the surface area, and the type of compound encapsulated.

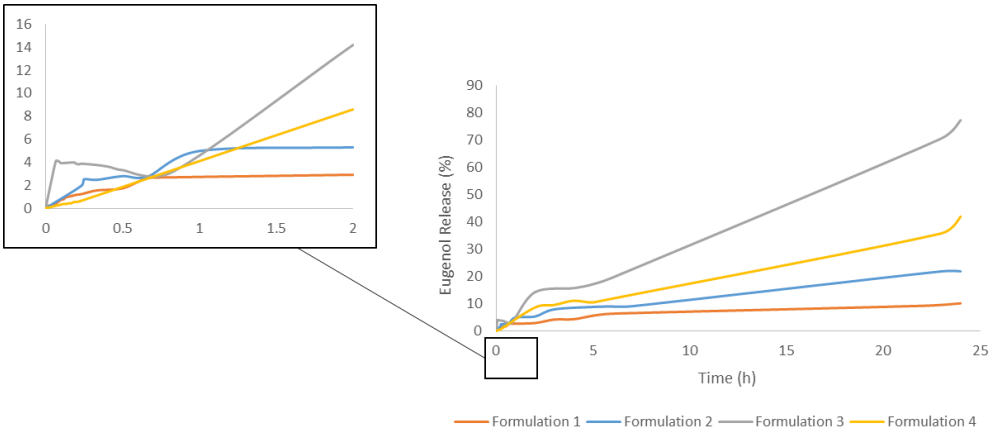


Figure 11 - Results of the sustained release study in 24 hours, at room temperature, in UPW (pH 5.6), for the four microparticles formulations

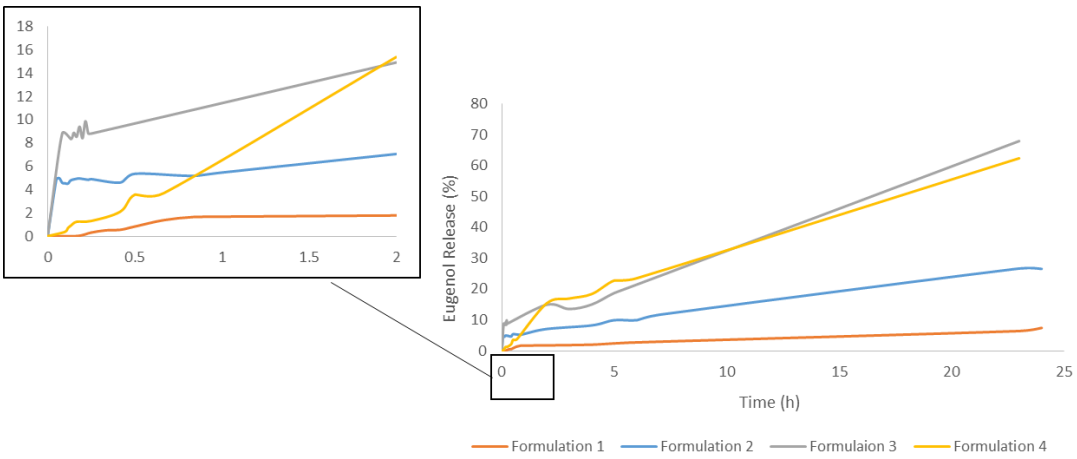


Figure 12 - Results of the sustained release study in 24 hours, at room temperature, in Basic Medium (pH 9), for the four microparticles formulations

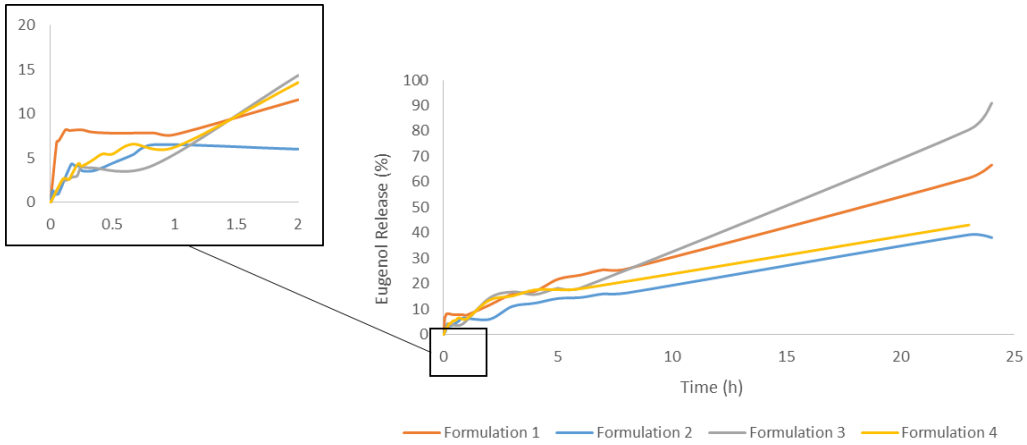


Figure 13 - Results of the sustained release study in 24 hours, at room temperature, in Acid Medium (pH 2), for the four microparticles formulations

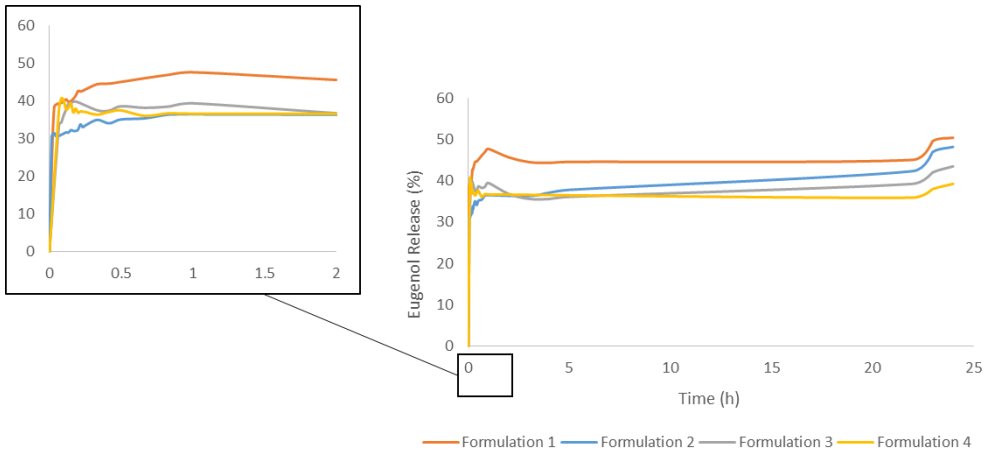


Figure 14 - Results of the sustained release study in 24 hours, at room temperature, in OCT, for the four microparticles formulations

Finally, these preliminary release tests in ultra-pure water and octanol indicated that depending on the cosmetic application, aqueous or oily formulations, ethyl cellulose microparticles with entrapment of eugenol by double emulsion solvent could be used. Similarly, preliminary tests using ultra-pure water with HCl and $NaHCO_3$ (hydrochloric acid and sodium bicarbonate, respectively) allowed the conclusion that the obtained delivery systems could also be used in food products. Depending on the intended release, if is expected to be in the stomach (acidic environment) or in the intestine (basic environment), those different mediums were recreated.

6 CONCLUSIONS

In this study eugenol-based ethyl cellulose microparticles were prepared by the double emulsion solvent evaporation method in order to be applied in industrial sectors. At far as it is known, this was the first study to successfully report a double emulsion solvent evaporation technique for eugenol encapsulation into ethyl cellulose microparticles. The particles were characterized by encapsulation efficiency, product yield, water content, particle size and size distribution, surface morphology, as well as the sustained release profile. Regarding the results about these parameters, the maximum product yield was 82.4 $\pm$ 2.6% in F2; the maximum encapsulation efficiency was 93.3 $\pm$ 0.2% in F3; and the water content was found out to be maximum in formulation 4 (87.5 ± 0.6%). Additionally, the prepared microparticles showed sizes ranging between 128.0 ± 0.2 μm and 850.0 ± 19.1 μm for the overall formulations tested, and the lower SPAN value obtained was 1.8 $\pm$ 0.1 for F2. The microparticles were generically spherical, polydisperse, porous and superficially rough. Moreover, an UV-Vis Spectrometry method for eugenol determination and quantification was developed and validated. Generally, all parameters were within the conditions proposed, indicating that this method was sensitive, linear, and precise, with low detection and quantification limits. The proposed method was used to predict the encapsulation efficiency and the release profile of eugenol from the final particles, in the four mediums investigated. In addition, this study investigated the sustained release profile of eugenol under simulated topical conditions, namely in hydrophilic and lipophilic matrices, as well as under simulated gastrointestinal conditions, namely gastro and intestinal fluids. The release was much faster when hydrochloric acid was present in the solution comparing to when sodium bicarbonate was added. Regarding the ultra-pure water and octanol medium, eugenol microparticles in octanol showed a faster release profile compared to release in ultra-pure water. For instance, F3 was the one showing highest final accumulated releases after 24 hours in ultra-pure water (77%), basic medium (68%) and acid medium (91%), while in octanol the highest final accumulated releases after 24 hours was found out for F1 (50%). We might assume that the formulation parameters used in F3 could be our optimized conditions because those particles obtained the higher release profile in almost all the formulations (exception in octanol) allowing its application in the recreated cosmetic and food formulations.

These preliminary tests reveal the potential of ethyl cellulose microsystems prepared by the double emulsion solvent evaporation technique for eugenol delivery and suggest that microparticles could be eligible vehicles to encapsulate eugenol and protect it, while maintaining its beneficial properties in order to be potential applied in industrial sectors.

7 LIMITATIONS AND RECOMMENDATIONS FOR FUTURE WORK

Despite the positive aspects of this study, some limitations should be highlighted. The availability of the equipment (coulter counter and freeze-dryer were shared with other researchers and students), and the fact that the UV-Vis spectrophotometer was broken, were the major limitations that delayed or influence the obtainment of results in the present project.

Microencapsulation for cosmetic and food industries still faces technological challenges, especially for the microencapsulation of sensitive bioactive compounds, such as essential oils. In fact, eugenol is a highly volatile component which normally leads to quicker releases, and due to that, its application has been challenged and further researches need to be accomplished. The release profile of compounds from microparticles could be affected by several factors and, therefore, future work should investigate and optimize these parameters to obtain a system with the intended properties. Additionally, further studies on formulation parameters and conditions, as well as alternative materials and techniques should be carried-out to obtain optimized microparticles that would possibly control the release of eugenol oil more effectively. Firstly, because for cosmetic applications a slower release is intended and, secondly, because for food formulations, it is important to consider which organ is supposed to be the delivery host of the active ingredient. Nevertheless, as preliminary tests, the results of this present study suggested the success of eugenol microencapsulation, suggesting the need to explore micron-sized delivery systems for eugenol topical and internal body delivery.

Sustained release experiments could be further tested within the incubator (37 ºC) in order to recreate the human body temperature, or for longer time, in order to better understand how volatile compounds like eugenol would behave. The analysis of the antimicrobial activity of encapsulated eugenol against selected cell lines would provide relevant information on the potential of eugenol microsystems, and so would the evaluation of it anti-antioxidant capacity. It would be also interesting in future work to attempt and develop a suitable extraction method for phenolic compounds, including eugenol, from laurel plant (*Laurus nobilis*) or other natural sources of this compound.

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Appendix

Analytical Method Validation

A1 - Validation of the UPW medium (pH 5.6): Repeatability analysis

Same day measurement	Mean[a] (M ± s)	RSD (%)
Low Concentration (4mg/L)	0.054 ± 0.0	1.1
Intermediate Concentration (8mg/L)	0.120 ± 0.0	0.4
High Concentration (18mg/L)	0.307 ± 0.0	0.2

UPW - Ultra-pure water; M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A2 - Validation of the UPW medium (pH 5.6): Intermediate precision analysis

Measurements on 3 different days	Low Concentration (4mg/L)	Intermediate Concentration (8mg/L)	High Concentration (18mg/L)
Mean [a] Day 1 (M ± s)	0.072 ± 0.0	0.128 ± 0.0	0.320 ± 0.0
RSD Day 1 (%)	0.3	0.7	0.3
Mean [a] Day 2 (M ± s)	0.061 ± 0.0	0.127 ± 0.0	0.280 ± 0.0
RSD Day 2 (%)	1.4	0.4	0.1
Mean [a] Day 3 (M ± s)	0.063 ± 0.0	0.129 ± 0.0	0.275 ± 0.0
RSD Day 3 (%)	0.5	0.8	0.5

UPW - Ultra-pure water; M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions.

A3 - Validation of the basic medium (pH 9): Repeatability analysis

Same day measurement	Mean[a] (M ± s)	RSD (%)
Low Concentration (2 mg/L)	0.039 ± 0.0	1.3
Intermediate Concentration (8 mg/L)	0.133 ± 0.0	0.3
High Concentration (12 mg/L)	0.196 ± 0.0	0.3

M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A4 - Validation of the basic medium (pH 9): Intermediate precision analysis

Measurements on 3 different days	Low Concentration (2 mg/L)	Intermediate Concentration (8 mg/L)	High Concentration (12 mg/L)
Mean [a] Day 1 (M ± s)	0.042 ± 0.0	0.149 ± 0.0	0.205 ± 0.0
RSD Day 1 (%)	0.9	0.5	0.2
Mean [a] Day 2 (M ± s)	0.055 ± 0.0	0.096 ± 0.0	0.203 ± 0.0
RSD Day 2 (%)	3.1	0.6	0.3
Mean [a] Day 3	0.025 ± 0.0	0.073 ± 0.0	0.203 ± 0.0
RSD Day 3 (%)	2.3	6.6	9.0

M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A5 - Validation of the acid medium (pH 2): Repeatability analysis

Same day measurement	Mean[a] (M ± s)	RSD (%)
Low Concentration (2 mg/L)	0.029 ± 0.0	2.4
Intermediate Concentration (14 mg/L)	0.217 ± 0.0	0.4
High Concentration (30 mg/L)	0.465 ± 0.0	0.3

M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A6 - Validation of the acid medium (pH 2): Intermediate precision analysis

Measurements on 3 different days	Low Concentration (2 mg/L)	Intermediate Concentration (14 mg/L)	High Concentration (30 mg/L)
Mean [a] Day 1 (M ± s)	0.030 ± 0.0	0.216 ± 0.0	0.467 ± 0.0
RSD Day 1 (%)	5.2	0.7	0.3
Mean [a] Day 2 (M ± s)	0.044 ± 0.0	0.230 ± 0.0	0.474 ± 0.0
RSD Day 2 (%)	3.0	0.8	0.2
Mean [a] Day 3 (M ± s)	0.040 ± 0.0	0.248 ± 0.0	0.463 ± 0.0
RSD Day 3 (%)	2.6	0.3	0.1

M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A7 - Validation of the OCT medium: Repeatability analysis

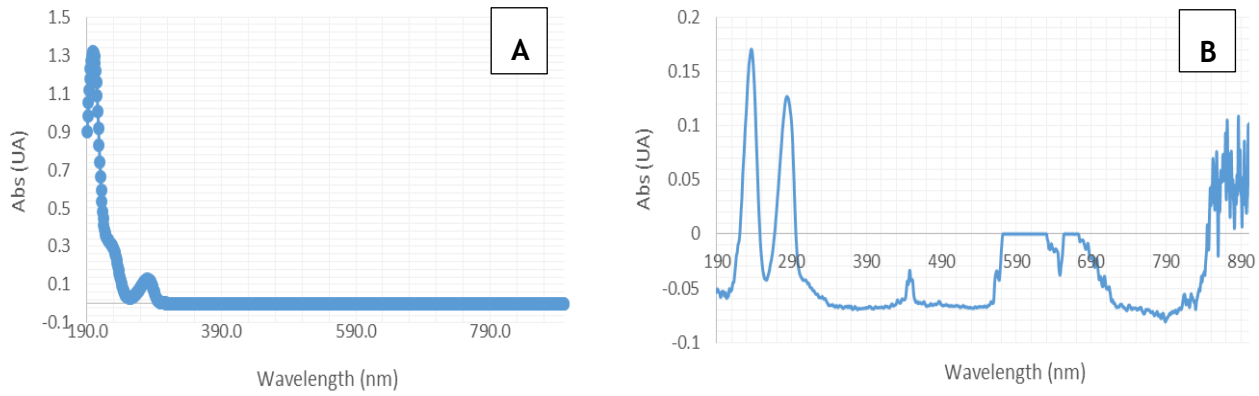
Same day measurement	Mean[a] (M ± s)	RSD (%)
Low Concentration (0.5 mg/L)	0.001 ± 0.0	0.0
Intermediate Concentration (10 mg/L)	0.216 ± 0.0	3.3
High Concentration (20 mg/L)	0.454 ± 0.0	1.1

OCT - Octanol; M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 6 repetitions.

A8- Validation of the OCT medium: Intermediate precision analysis

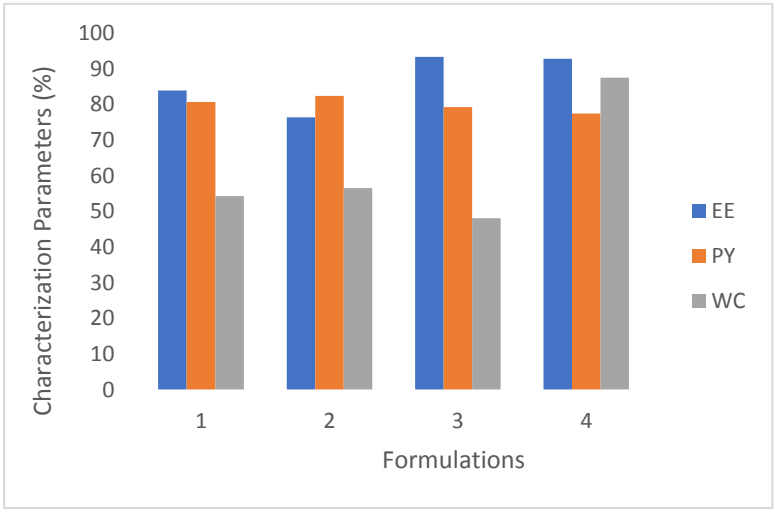
Measurements on 3 different days	Low Concentration (0.5 mg/L)	Intermediate Concentration (10 mg/L)	High Concentration (20 mg/L)
Mean [a] Day 1 (M ± s)	0.005 ± 0.0	0.220 ± 0.0	0.457 ± 0.0
RSD Day 1 (%)	0.0	2.8	0.9
Mean [a] Day 2 (M ± s)	0.053 ± 0.0	0.302 ± 0.0	0.541 ± 0.0
RSD Day 2 (%)	8.5	1.5	0.1
Mean [a] Day 3 (M ± s)	n.f.	0.238 ± **0.0**	0.462 ± **0.0**
RSD Day 3 (%)	n.f.	0.3	0.5

OCT - Octanol; M - Mean value; s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions; n.f. - not found.



A9 - Absorption spectra of eugenol between 190 and 900 nm in ultra-pure water (A) and octanol (B)

Microparticles Characterization



A10 - Bars charts with the results obtained for the microparticles characterization parameters in the four different formulations: EE (encapsulation efficiency); PY (product yield); WC (water content)

Particle Morphology and Particle Size Distribution

A11 - Particle Size Distribution Analysis for F1

	SM1		SM6		SM7	
Parameters	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± SD)**	**RSD (%)**	**Mean [a] (M ± SD)**	**RSD (%)**
D10	32 ± 0.4	1.1	97 ± 3.1	3.2	41 ± 1.1	2.7
D50	77 ± 0.9	1.2	324 ± 4.9	1.5	110 ± 5.3	4.8
D90	367 ± 21.5	5.8	846 ± 95.0	11.2	246 ± 24.3	9.9
Mean (µm)	153 ± 6.0	3.9	426 ± 13.8	3.2	132 ± 14.8	11.2
SPAN	4.4 ± 0.2	5.5	2.3 ± 0.3	14.3	1.9 ± 0.1	7.1

s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions: F - Formulation.

A12- Particle Size Distribution Analysis for F2

	SM3		SM4		SM5	
Parameters	Mean[a] (M ± s)	RSD (%)	Mean[a] (M ± SD)	RSD (%)	Mean[a] (M ± SD)	RSD (%)
D10	40 ± 0.2	0.4	47 ± 2.4	5.2	48 ± 1.1	2.3
D50	91 ± 1.9	2.1	150 ± 6.2	4.1	107 ± 2.9	2.7
D90	219 ± 22.7	10.3	320 ± 13.2	4.1	1078 ± 10.9	1.0
Mean (µm)	118 ± 17.1	14.4	196 ± 18.6	9.5	306 ± 11.2	3.7
SPAN	2.0 ± 0.2	10.5	1.8 ± 0.1	6.2	9.7 ± 0.2	1.8

s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions; F - Formulation.

A13 - Particle Size Distribution Analysis for F3

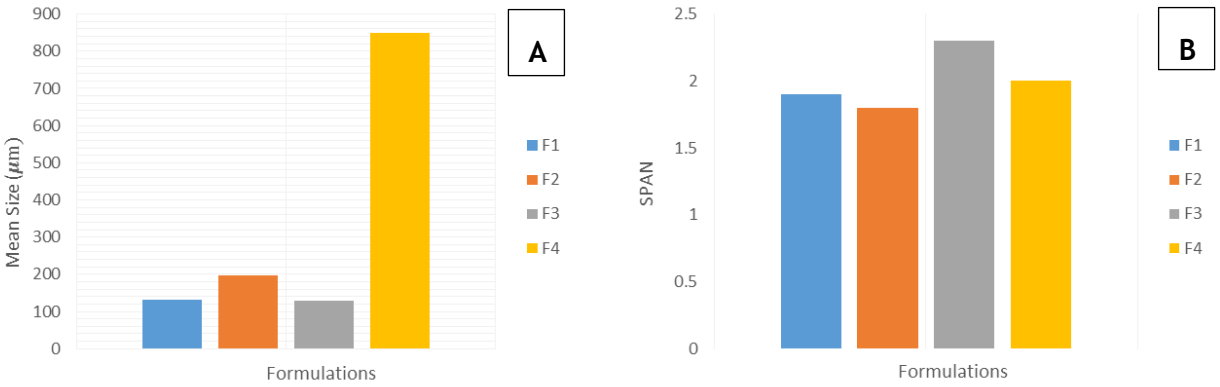
	SM8		SM9		SM10	
Parameters	Mean[a] (M ± s)	RSD (%)	Mean[a] (M ± SD)	RSD (%)	Mean[a] (M ± SD)	RSD (%)
D10	36 ±0.3	0.7	39 ± 0.1	0.2	68 ± 1.7	2.5
D50	88 ± 0.4	0.5	77 ± 1.9	2.5	173 ± 5.9	3.4
D90	237 ± 1.6	0.7	280 ± 1.8	0.7	754 ± 37.0	4.9
Mean (µm)	128 ± 0. 2	0.1	148 ± 4.5	3.0	289 ± 4.8	1.7
SPAN	2.3 ± 0.0	0.4	3.2 ± 0.1	2.1	4.0 ± 0.3	7.8

s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions; F - Formulation.

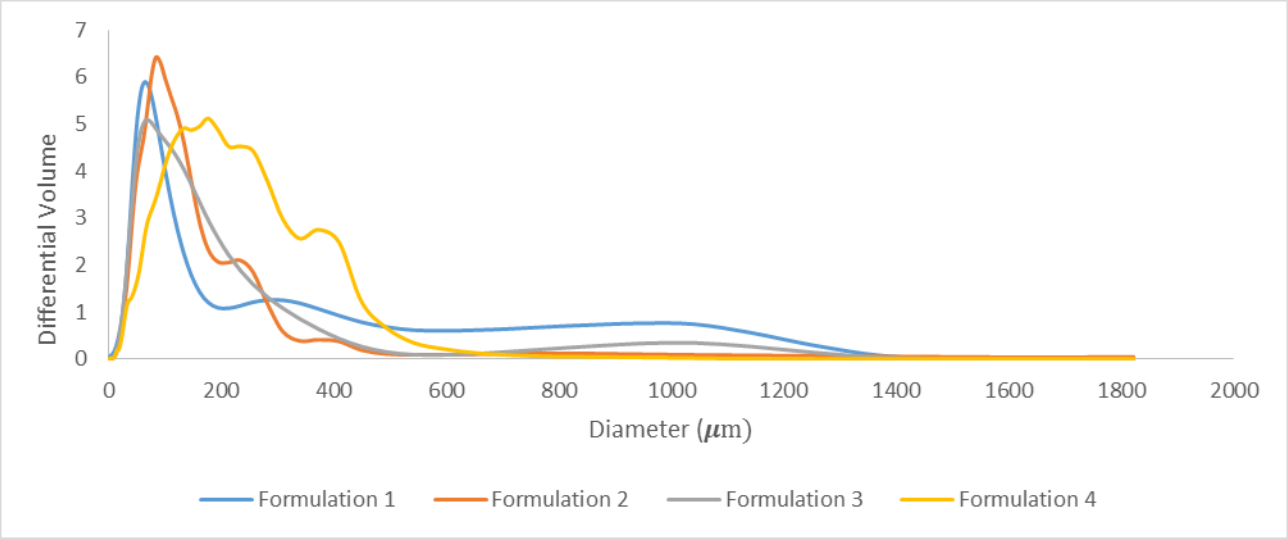
A14 - Particle Size Distribution Analysis for F4

	SM11		SM12		SM13	
Parameters	**Mean[a] (M ± s)**	**RSD (%)**	**Mean[a] (M ± SD)**	**RSD (%)**	**Mean[a] (M ± SD)**	**RSD (%)**
D10	32 ± 1.5	4.7	71 ± 1.4	1.9	49 ± 3.5	7.0
D50	99 ± 10.4	10.6	829 ± 32.7	3.9	150 ± 11.4	7.6
D90	392 ± 68.9	17.6	1740 ± 10.4	0.6	346 ± 37.5	10.9
Mean (µm)	174 ± 31.9	18.3	850 ± 19.1	2.3	177 ± 16.8	9.5
SPAN	3.6 ± 0.3	9.0	2.0 ± 0.1	3.3	2.0 ± 0.1	4.1

s - Standard deviation; RSD - Relative Standard Deviation; [a] - Mean of 3 repetitions, F - Formualtion.

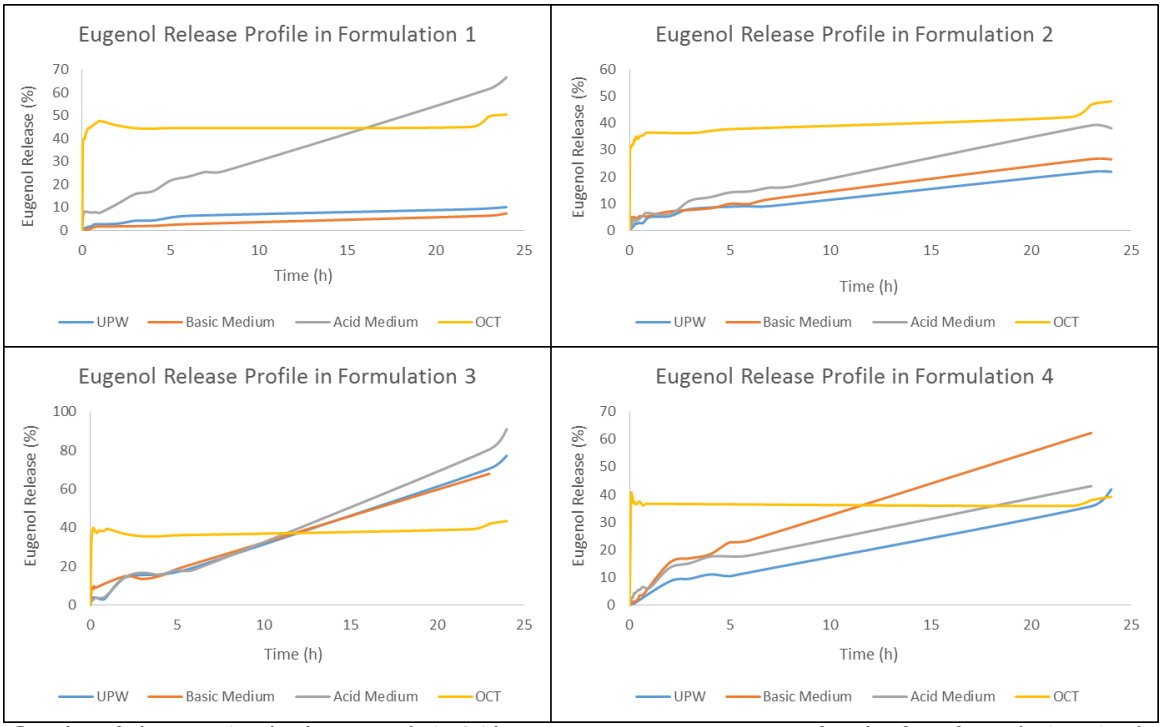


A15 - Bars charts with the results obtained for the microparticles mean size (A) and microparticles polydispersity degree (B) in the four different formulations



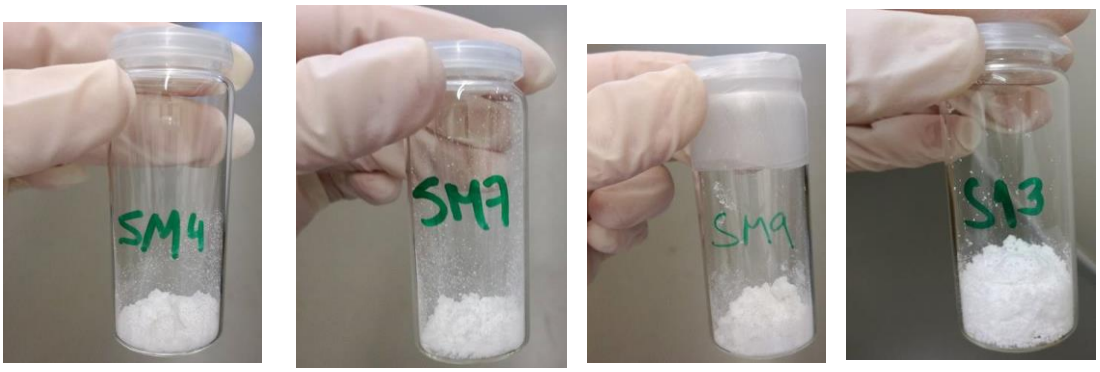
A16 - Examples of particles sizes distributions obtained in the four different formulations according to differential volume

Sustained release studies



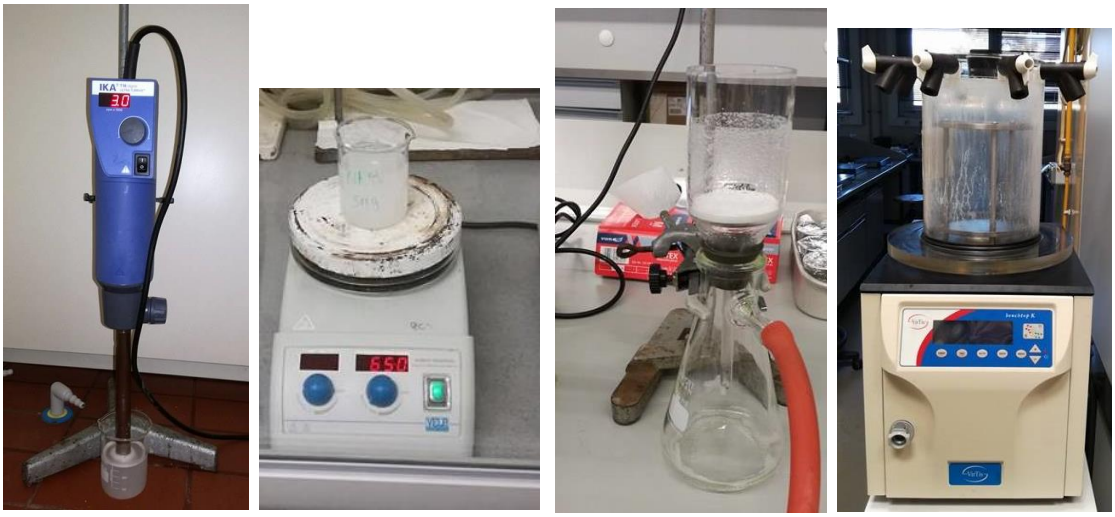
A17 - Results of the sustained release study in 24 hours, at room temperature, for the four formulations in the four different mediums: UPW, Basic Medium, Acid Medium and OCT

Photographs of the Practical Procedure used in the Laboratory



A18 - Final microparticles obtained in each individual experiment after the filtration step (examples of the four formulations, between 1 and 4, from left to right)

A19 - Example of microparticles powder obtained after the filtration step



A20 - Main equipment used for the production of microparticles in the present study (high-pressure homogenizer; stirring plate; vacuum filtration system; and freeze-dryer (from left to right)