Integrated Master in Bioengineering- Specialization in Biological Engineering

Strategies of microencapsulation of analgesics: The case study of Acetylsalicylic Acid

Master's Thesis

of

Ana Mónica Campos Mota

Developed within the discipline of Dissertation

Conducted at

Laboratory for Process Engineering, Environment, Biotechnology and Energy

Supervisor: **Prof. Lúcia Santos**

Department of Chemical Engineering

June, 2018

"Success depends in a very large measure upon individual initiative and exertion, and cannot be achieved except by a dint of hard work."

Anna Pavlova

Acknowledgments

I would like to express my very appreciation to all those who supported me in any way during this work.

First of all, I would like to thank my supervisor, Dr. Lúcia Santos, for giving me the opportunity to follow such an interesting topic and also for her help and guidance throughout the project. I am grateful for your support, availability, criticism and comprehensive advises.

For Eng. Filipa Paulo, for being my central pillar in this project. For her, before all the others, I owe my most sincere thanks for supporting myself daily, for the extra hours in the laboratory, for accompanying me on this journey and for all the availability and dedication given. Whenever necessary, I knew how to advise myself and how to criticise myself, as always and in everything in life. For the joys, dismay, anguish and especially for all understanding, thank you "mãezinha".

I am thankful to Faculty of Engineering of the University of Porto (FEUP), Department of Chemical Engineering (DEQ), and Laboratory for Process Engineering, Environment, Biotechnology, and Energy (LEPABE), for allowing me to use all the required facilities and resources for this thesis. I also would like to express my gratitude to the whole 201 group laboratory for receiving me so well and for the excellent atmosphere they have given me.

This work was financially supported by the projects POCI-01-0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy – UID/EQU/00511/2013) funded by the European Regional Development Fund (ERDF), through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) by national funds, through FCT - Fundação para a Ciência e a Tecnologia and by the project NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION, supported by North Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

For my friends in Biological Engineering, I would like to express my thanks for the company, leisure time and for being present during this stage of my life.

To my boyfriend, thank you for patience, love and for always believing in me, for sharing with me all difficulties and complaints but also the small victories, enthusiasm and confidence.

Finally, for the support, for investing and believing in me, for love and encouragement, I would like to thank my parents Carlos Mota and Irene Campos and my sister Daniela Mota.

Abstract

This work focuses on to find innovative systems of controlled release of drugs that allow to obtain medicines adapted to the mode of administration, reducing the side effects, allowing a specific action of the drug and increasing its compliance by the patient. Some therapeutic agents are chemically unstable and, therefore, being rapidly hydrolyzed or enzymatically degraded *in vivo*, requires multiple administrations. Traditional therapies have been progressively replaced by technologies for controlled drug delivery, such as microencapsulation. Microencapsulation arises in the context of controlled drug delivery systems, since different techniques allow to protect the therapeutic agent from hydrolytic and/or enzymatic degradation, among other possible reactions, allowing it to be released over time. The main objective of this project was the study of microencapsulation strategies of acetylsalycilic acid, due to its high consumption (50 billion aspirin tablets are consumed each year throughout the world) , encapsulating it with 3 different polymers (ethylcellulose, polycaprolactone and poly(lactic-co-glycolic acid)). The acetylsalicylic acid was microencapsulated using different emulsification methods ($w_1/o/w_2$ and s/o/w). Only the microparticles resulting from the $w_1/o/w_2$ emulsion, were characterized according to encapsulation efficiency, product yield, loading, particle size distribution and morphology, because the results for the remaining emulsion were not as expected and therefore were eliminated. Controlled release studies were performed on simulated gastrointestinal fluids. The analytical method was developed and validated. Regarding the characterization parameters obtained, the product yield varied between 70.3 ± 14.5% and 98.3 ± 3.0%; the encapsulation efficiency between 89.1 ± 0.6% and 99.6 ± 0.3%; and the loading varied between 3.7 ± 0.4% and 5.5 ± 1.2%. The microparticles obtained with poly(lactic-co-glycolic acid) were those that obtained the highest efficiency of encapsulation, whereas the microparticles coated with ethylcellulose were the ones that obtained the highest product yield. Regarding loading, polycaprolactone microparticles obtained the highest percentage. The prepared microparticles presented sizes varying from 27.6 ± 3.1 µm to 53.4 ± 17.8 µm for the overall formulations tested, being generally spherical, monodisperse, few porous and superficially smooth. The microparticles obtained with poly(lactic-co-glycolic acid) showed the lowest polydispersity and particle size. The highest percentages of release at 2 hours were for poly(lactic-co-glycolic acid) encapsulated microparticles: 1.8% in simulated gastric fluid and 9.4% in simulated intestinal fluid.

Keywords: Microencapsulation, acetylsalicylic acid, controlled release, microparticles, double emulsion by solvent evaporation, ethylcellulose, polycaprolactone, poly (lactide-co-glycolide acid)

Resumo

Este trabalho tem como objetivo encontrar sistemas inovadores de libertação controlada de fármacos que permitam obter medicamentos adaptados ao modo de administração, reduzindo os efeitos colaterais, permitindo uma ação específica do fármaco e aumentando a sua adesão no paciente. Alguns agentes terapêuticos são quimicamente instáveis e, portanto, sendo rapidamente hidrolisados ou degradados enzimaticamente *in vivo*, requerem múltiplas administrações. As terapias tradicionais foram progressivamente substituídas por tecnologias para administração controlada de fármacos, como a microencapsulação. A microencapsulação surge no contexto de sistemas de libertação controlada de fármacos, pois diferentes técnicas permitem proteger o agente terapêutico da degradação hidrolítica e/ou enzimática, entre outras possíveis reações, permitindo a sua libertação ao longo do tempo. O principal objetivo deste projeto foi o estudo das estratégias de microencapsulação do ácido acetilsalicílico, devido ao seu alto consumo (50 biliões de caixas de aspirina são consumidas por ano, em todo o mundo), encapsulando-o com 3 diferentes polímeros (etilcelulose, policaprolactona e ácido poli (lático-co-glicólico)). O ácido acetilsalicílico foi microencapsulado usando diferentes métodos de emulsificação ($w_1/o/w_2$ e s/o/w). Somente as micropartículas resultantes da emulsão $w_1/o/w_2$ foram caracterizadas quanto à sua eficiência de encapsulação, rendimento do produto, *loading*, distribuição de tamanho e morfologia, pois os resultados para a restante emulsão não foram os esperados e, portanto, foram eliminados. Estudos de libertação controlada foram realizados em fluídos gastrointestinais simulados. O método analítico foi desenvolvido e validado. Em relação aos parâmetros de caracterização obtidos, o rendimento do produto variou entre 70,3 ± 14,5% e 98,3 ± 3,0%; a eficiência de encapsulação entre 89,1 ± 0,6% e 99,6 ± 0,3%; e o *loading* variou entre 3,7 ± 0,4% e 5,5 ± 1,2%. As micropartículas obtidas com o ácido poli (lático-co-glicólico) foram as que obtiveram maior eficiência de encapsulação, enquanto que as micropartículas revestidas com etilcelulose foram as que obtiveram maior rendimento de produto. Relativamente ao *loading*, as micropartículas de policaprolactona obtiveram a maior percentagem. As micropartículas preparadas apresentaram tamanhos de partícula variando de 27,6 ± 3,1 μm a 53,4 ± 17,8 μm para as formulações globais testadas, sendo geralmente esféricas, monodispersas, pouco porosas e superficialmente suaves. As micropartículas obtidas com ácido poli (láctico-co-glicólico) apresentaram a menor polidispersividade e tamanho de partícula. As maiores percentagens de libertação em 2 horas foram para as micropartículas encapsuladas com o ácido poli (láctico-co-glicólico): 1,8% em fluído gástrico simulado e 9,4% em fluído intestinal simulado.

Palavras-chave: Microencapsulação, ácido acetilsalicílico, libertação controlada, micropartículas, dupla emulsão por evaporação do solvente, etilcelulose, policaprolactona, ácido poli (lático-co-glicólico)

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

25th of June, 2018

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(Ana Mónica Campos Mota)

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Glossary

APC	Active pharmaceutical compound
ASA	Acetylsalicylic acid
Abs	Absorbance
C	Concentration
CAP	Cellulose acetate phthalate
CAS	Chemical abstracts service
CMC	Carboxymethylcellulose
COX	Cyclooxygenase
CV	Coefficient of variation
d_{10}	10 percent of particle size distribution lies below this value
d_{50}	Median of particle size distribution
d_{90}	90 percent of particle size distribution lies below this value
DCM	Dichloromethane
DDS	Drug delivery system
DE	Double emulsion
DEQ	Departamento de Engenharia Química
DL	Drug loading
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
EC	Ethylcellulose
EE	Encapsulation efficiency
Eq	Equation
FDA	American Food and Drug Administration
FEUP	Faculdade de Engenharia da Universidade do Porto
IUPAC	International Union of Pure and Applied Chemistry
LM	Microscopy of light

LEPABE	Laboratory for Process Engineering, Environment, Biotechnology and Energy
LOD	Limit of detection
LOQ	Limit of quantification
MPs	Microparticles
NPs	Nanoparticles
NSAIDs	Non-steroidal anti-inflammatory drugs
O	Oil phase
OWR	Oil-in-water ratio
PCL	Polycaprolactone
PCT	Paracetamol
PLA	Polylactide
PLGA	Poly(lactide-co-glycolide acid)
PVA	Polyvinyl alcohol
PEG	Polyethylene glycol
PY	Product yield
Rpm	Rotations per minute
SEM	Scanning electron microscope
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SLMs	Solid lipid microparticles
SSF	Simulated salivary fluid
UPW	Ultrapure water
USD	United States Dollar
UV	Ultraviolet radiation
w_1	Internal aqueous phase
w_2	External aqueous phase

1 Background motivation and project guideline

1.1 Background motivation

Therapeutics agents currently on the market can be place into either one of 4 categories: small molecules, biotherapeutics, natural products and nucleic-acid-based therapeutics (Gad 2012). However, some therapeutic agents are chemically unstable and, therefore, being hydrolysed rapidly, involve multiple administrations and consequently a high dosage amount. Also, they cause adverse effects, such as urinary retention, slow breathing, liver problems, gastritis, among others (Carter et al. 2014). Since analgesics are a type of therapeutic agent, these are the best-sold group of drugs in Portugal (Infarmed 2016), and they are part of people's daily lives to treat headaches, muscle aches, toothaches, among others. Presently, pharmaceutical research seeks to find new and innovative drug delivery systems (DDS), in order to obtain pharmaceutical products to reach the market associated with specific goals such as the reduction of adverse reactions and side effects, being suitable for administration mode, allowing site-specific delivery, improving the shelf-life and patient compliance (Agnihotri et al. 2012). An efficient DDS is the one that allows the active pharmaceutical compound (APC) to reach the target site, in the required time and for the desired time. Four major factors are considered to achieve an efficient DDS: administration route, pattern of APC release, method of delivery and production process also known as formulation process. Thus, microencapsulation technology arises in the context of controlled DDS because its various techniques allow to protect the therapeutic agent from rapid hydrolytic and/or enzymatic degradation, potential oxidation, among other possible reactions, allowing it to be controlled to achieve the desired concentration over time. It is also suitable to mask the bitter taste of various drugs, reduce irritations in the gastrointestinal tract and also the odour, separate incompatible substances and provide protection to substances to encapsulate against atmospheric effects (Brasileiro 2011).

Microencapsulation is a set of techniques in which substances in the three states of matter (solid, liquid, and gaseous) are coated by an encapsulating agent, resulting in particles having microscopic dimensions. This technology enables liquid and gaseous phase materials to be easily manipulated such as the solids, and thus provide some measure of protection for those handling hazardous materials (Dubey et al. 2009). Microencapsulation has been studied and used in several industrial areas (food industries, cosmetics, textiles, agriculture, electronics and biomedical), mainly in the pharmaceutical sector, which has allowed the development of controlled drug release formulas that have the ability to release the active agent only in the place or organ where it should (Paulo & Santos 2016). There are numerous possibilities of using

microencapsulation as a technique to obtain products with high added value and, therefore, widespread interest has led to the development of microencapsulation technology. Several studies published in the area of microencapsulation indicate that industrial and academic sectors are focused on the exploration of this area, especially in the pharmacological field.

1.2 Aims of the thesis

Acetylsalicylic acid is a high consumption analgesic (Jones 2005) (50 billion aspirin tablets are consumed each year throughout the world) and is used to alleviate mild to moderate pain, however, taking it can cause problems in the gastrointestinal tract, and in order to solve this problem, this project aims to formulate microspheres for a controlled DDS, encapsulating acetylsalicylic acid (ASA) by double emulsion (DE) solvent evaporation technique using three biodegradable polymers (Ethylcellulose (EC), Polycaprolactone (PCL), and Poly(lactic-co-glycolic acid) (PLGA)) and different emulsification methods ($w_1/o/w_2$ and s/o/w). Only the microparticles resulting from the $w_1/o/w_2$ emulsion were used to investigate the influence on the formulation parameters of microspheres, because the results for the other emulsion were not as expected and therefore were eliminated. In this study it is also presumed to accomplish the release studies of ASA in three different mediums: salivary, gastric and intestinal fluids simulated in order to recreate the gastrointestinal tract (Figure 1). Furthermore, the influence of selected parameters on the final characteristics of the microparticles (encapsulation efficiency, product yield, loading, distribution of shape and particle size) was studied through microencapsulation formulations. Moreover, the project aims to develop and validate the analytical method (UV-Vis Spectrophotometry) for acetylsalicylic acid determination and quantification and to determine performance parameters such as quantification parameters (linearity, sensitivity and limits of detection and quantification).

1.3 Thesis organization

This document is divided into eight chapters and their respective sub-chapters. In Chapter 1, a Background section provides a general perspective of the problem under study highlighting some reasons for the study of the thesis subject. Additionally, presents the aims of the thesis and the thesis organization. Chapter 2 is devoted to a presentation, review and explanation of theoretical concepts needed for the comprehension and presentation of this this project results: it is made a presentation about analgesics, giving special emphasis to ASA. It follows the description of microencapsulation technology, and their application in pharmaceutical industry. The DE solvent evaporation technique is discussed in more detail and is presented the parameters affecting microspheres properties. It is briefly discussed the mechanism of controlled release and encapsulating agents. Chapter 3 provides a review on the state of the art about microencapsulation of active pharmaceutical compound: acetylsalicylic acid. Chapter

4 describes the materials and methods for the formulation and characterization of microspheres. In Chapter 5 the results and discussion are presented. Chapter 6 presents the main conclusions of this project. In Chapter 7 is indicated the limitations, possible future relevant work within the topic and a final appreciation. Additional data is presented in the Appendix sections (from Appendix A to Appendix D).

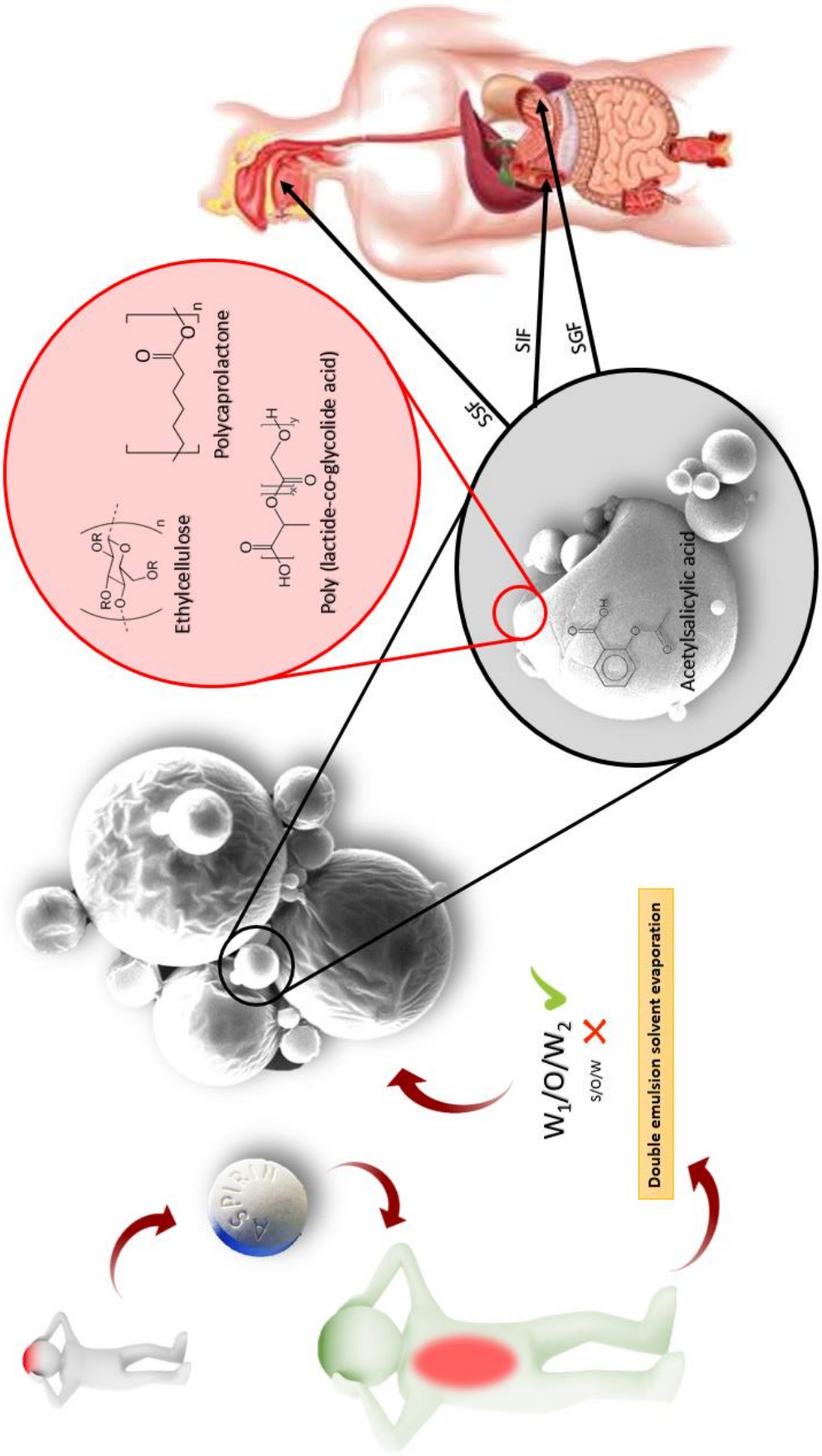


Figure 1 – Graphical abstract of the aim of this dissertation regarding acetylsalicylic acid microencapsulation

2 Introduction

2.1 Pharmacological Compounds

The use of analgesics for the treatment of pain dates back to the 18th century, where the infusion of plants such as *Salix alba vulgaris* was performed to obtain the desired effects. Through certain discoveries, the introduction of new techniques and products was initiated, initiating the therapeutic intervention of important compounds of analgesic, antipyretic and anti-inflammatory action, which continue in development until the present day (Silva 2002).

Pain encompasses physiological, psychological, cognitive and affective aspects, as well as being influenced by cultural and social factors that act on the behavioral reaction of the individual to the pain. The sensation of pain is related to the perception of the nervous system, being natural to all people, which characterises a personal experience of each subject. The definition of pain, proposed by the International Association for the Study of Pain, is "an unpleasant sensory and emotional experience associated with a tissue injury, effective or potential, or described in terms of such injury." A painful stimulus causes the activation of pain fibres, causing chemical irritation or mechanical deformation of the nerve endings, resulting in depolarization of the pain fibres. The pain impulse is triggered by the first mechanical dysfunction of the lesion and is followed by irritation due to the inflammatory process (Starkey 2001). There are specific medications that are indicated to promote pain and inflammation, and are grouped and delimited into classes.

Analgesic and anti-inflammatory drugs are classified into cycloxygenase (COX) inhibitory drugs, phospholipase A2 inhibitory drugs, drugs that directly depress the nociceptor and central-acting drugs (Rang & Voeux 2004). Among the class of COX inhibitors are non-steroidal anti-inflammatory drugs (NSAIDs), ASA, paracetamol (PCT), nimesulide, meloxicam and diclofenac sodium. Among drugs belonging to the class of phospholipase A2 inhibitory drugs, corticosteroids or also called glucocorticoids may be indicated. Finally, the drugs belonging to the class of drugs that directly depress the nociceptor are dipyrone and diclofenac sodium (Fernandes 2006).

Analgesics are a diversified group of drugs that decrease or interrupt nerve transmission pathways, reducing the perception of pain.

Non-steroidal anti-inflammatory drugs make up the most commonly used class of medications among all therapeutic agents. Currently, there are more than 50 distinct types of NSAIDS in the market. These drugs are often indicated for the treatment of pain associated with inflammation and tissue injury, acting on the inhibition of the synthesis of prostaglandins

that are endogenous intermediates of the inflammatory process, thus acting on the musculoskeletal system (Howland et al. 2007).

Non-steroidal anti-inflammatory drugs have three main actions: anti-inflammatory, due to the reduction of prostaglandins; analgesic effect related to decreased prostaglandin production; and antipyretic effect, due to the decrease of the mediator prostaglandin, responsible for the elevation of the hypothalamic setpoint that exerts control over the temperature in the fever (Fernandes 2006). The anti-inflammatory action of NSAIDs is clearly related to inhibition of COX 2, usually resulting in vasodilation, pain, and indirectly in oedema.

Currently, the most common anti-inflammatories include ASA, diclofenac (sodium and potassium), ibuprofen, naproxen, indomethacin, ketoprofen, mefenamic acid, piroxicam and celecoxib. These drugs, when misused, can cause various problems, adverse reactions or side effects. In addition, they also cause direct aggression in the mucosa of the digestive tract, which occurs predominantly during absorption, because most drugs are acidic and acidic substances tend to accumulate intracellularly in areas of the body where the extracellular pH is low. Thus, the lower the acidity of a drug (higher pKa value) and the higher its rate of absorption and bioavailability, the lower the tendency to have direct effects on the mucosa of the digestive tract (Fernandes 2006).

2.1.1 Acetylsalicylic Acid

Acetylsalicylic acid is a derivative of salicylic acid and is the most widely used salicylate, considered in the group of analgesics, antipyretics and non-steroidal anti-inflammatory drugs (Fernandes 2006).

This compound is almost given orally. It is rapidly absorbed in the gastrointestinal tract, partly in the gastric mucosa, but mainly in the small intestine due to the best characteristics of absorption of this mucosa (Schrör 2016).

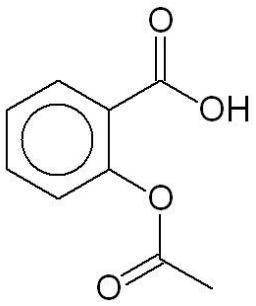
The ASA has a half-life of 15 to 20 minutes because it is converted to salicylic acid (Figure A1, Appendix A: Acetylsalicylic acid Synthesis Scheme) by esterases present in the intestinal wall, blood and liver. It irreversibly inhibits COX-1 and COX-2 and, therefore, presents a wide range of pharmacological actions. Acetylsalicylic acid causes ulceration, epigastric distress, haemorrhage because it has a direct irritant effect on gastric mucosa due to inhibition of prostaglandins and prostacyclins (Dash et al. 2010). It is a drug of great clinical utility that is used for its analgesic, antipyretic and anti-inflammatory action and also for its anticoagulant action.

This drug is used as an analgesic in the treatment of somatic pain (musculoskeletal pain) and a variety of other painful conditions, including a headache, migraine and dysmenorrhea. It is very useful in controlling pain associated with inflammatory processes and rapidly decreases

the increase in body temperature due to infection, tissue injury, or other disease states. It does not affect body temperature and does not reduce the temperature rise due to excessive exercise or ambient heat (Fernandes 2006).

Acetylsalicylic acid is formally known as acetylsalicylic acid. It is a crystalline powder with a slightly bitter taste (Table 1).

Table 1 - Physical and chemical properties of acetylsalicylic acid (Adapted from(PubChem 2005)*)*

Compound	Acetylsalicylic Acid
IUPAC name	2-acetyloxybenzoic acid
CAS number	50-78-2
Molecular Formula	$C_9H_8O_4$
Chemical Structure	
Molecular Weight (g.mol^{-1})	180.16
Melting Point (ºC)	135
Steam Pressure (mmHg at 25 ºC)	2.52×10^{-5}
pKa	3.41
Log K_{ow}	1.19
Solubility in water at 25 ºC (mg.L^{-1})	4600
Maximum absorption wavelength (nm)	275

The intake of high doses of acetylsalicylic acid causes several metabolic changes. Salicylates dissociate oxidative phosphorylation, mainly in the skeletal muscle, which results in an increase in O_2 consumption and therefore in CO_2 production. As a result, breathing stimulation is observed. In addition, it also results in the appearance of a neurological condition known as salicylism and characterised by tinnitus, deafness, headache, dizziness, nausea and vomiting.

2.2 Microencapsulation

Microencapsulation is a process of encapsulating a material that contains an active compound in a polymer (encapsulating agent) to protect the active compounds from external factors permanently or temporarily (Casanova & Santos 2016). This results in small particles called microparticles. These particles have diameters between 1-1000 μm (Singh et al. 2010). The small size of these particles provides a large surface area that is available for adsorption/desorption, chemical reactions, light scattering and so on.

The advance of microencapsulation began with the preparation of capsules containing dyes in 1950 by Green and Schleicher (Barrett Green & Schle Cher 1956). These were incorporated into paper for copying purposes and substituted carbon paper. Nowadays this approach has been widely explored by the pharmaceutical, food, cosmetic, textile, agricultural, veterinary, chemical and biomedical industries. The field with the highest level of microencapsulation applications is the pharmaceutical sector (68%), followed by foods (13%) and cosmetics (8%) (Figure 2) (Kim et al. 2007). There are numerous possibilities of using microencapsulation as a technique to obtain products with high added value, and therefore the widespread interest has developed in microencapsulation technology.

The global microencapsulation market size was United States Dollar (USD) 5.54 billion in 2015 and is expected to reach USD 8.73 billion by 2020. Pharmaceutical was the most significant application, accounting approximately 70% of market revenue share in 2013. Growing demand for microencapsulation for controlled release of active ingredients and targeted drug delivery is expected to have a positive impact on the market. Pharmaceutical growth in emerging economies of India, China, and Brazil is expected to augment microencapsulation market over the forecast period. Emergence of nanotechnology and microtechnology in the pharmaceutical industry is expected to challenge market growth over the next years (Grand View Research 2017).

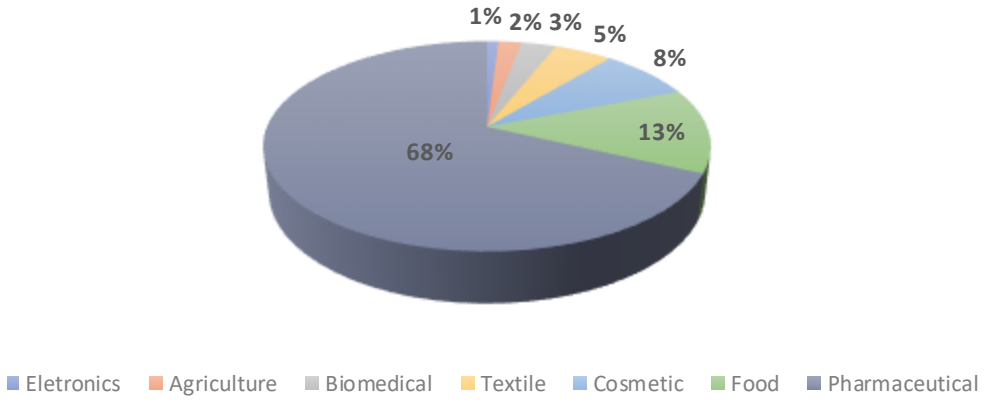


Figure 2 - Schematic representation of the statistical distribution of microencapsulation over different fields of application (Adapted from (Martins et al. 2014)*)*

Microencapsulation has many advantages comparing to non-encapsulated substances since it allows the protection and stabilisation of the core material and it is controlled, timed and targeted release. Product appearance and flow properties may also be improved, enhancing its handling, usage and storage. Undesirable organoleptic properties can be masked, and the evaporation of volatile ingredients can be controlled using microencapsulation. This technique can also be used to reduce the amount of ingredients in formulation being a cost-saving alternative (Casanova & Santos 2016).

The core materials in microcapsules may exist in the form of a solid, liquid or gas. The size of the core material plays an essential role in the diffusion, permeability and controlled release of the active compound. The polymer may be permeable, semipermeable or impermeable. The compatibility of the core material with the polymer is an essential criterion for increasing the efficiency of the microencapsulation.

The resulting products of microencapsulation techniques are designated microparticles (Figure 3). Microparticles can be distinguished in microspheres or microcapsules by their internal structure and morphology even though, the terms are often used synonymously (Herrero-Vanrell et al. 2014). The morphology of the internal structure of a microcapsule depends to a large extent on the polymer selected and the encapsulation method used. The microparticles can be classified as mononuclear, polynuclear or matrix type. The mononuclear microcapsules contain the polymer around the core and have a single hollow chamber within the capsule. The polynuclear microcapsules have many nuclei closed inside the shell so that they have some different sized chambers. The matrix-type microparticle has the active compounds integrated into the polymer matrix and distributed heterogeneously in the polymer matrix.

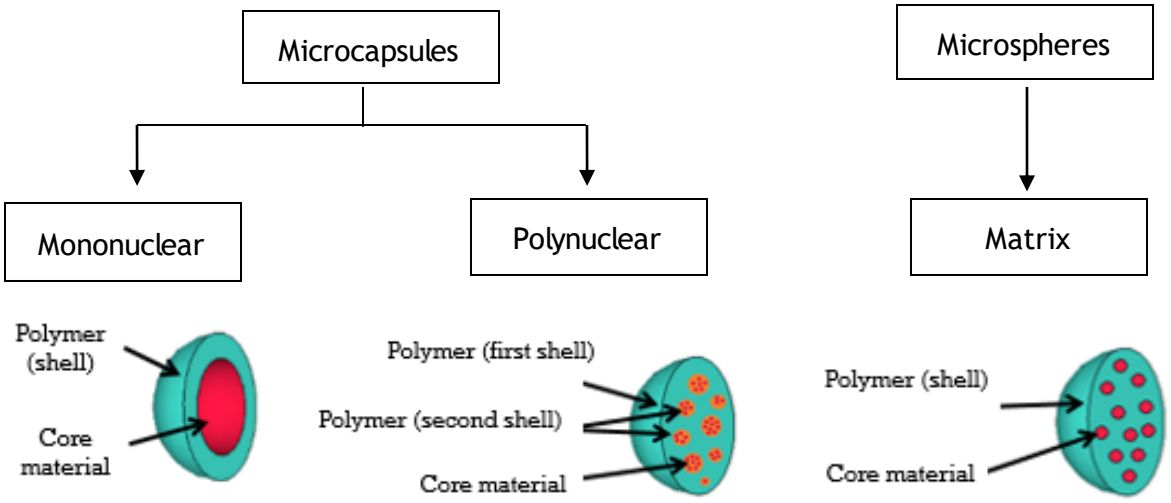


Figure 3 - Morphology of microparticles. (Adapted from (Paulo & Santos 2016)*)*

Microparticles are usually characterised by parameters such as particle size, encapsulation efficiency (EE), amount of encapsulated drug, the product yield (PY), ratio between the output mass obtained and the initial solid content of the feed solution, and loading, amount of the active agent present in the microparticles (Papadimitriou & Bikiaris 2009). The size and shape of the microparticles may be determined by light microscopy or by scanning electron microscopy (SEM or LM). The encapsulation efficiency (content of the effectively encapsulated core material) depends on several variables. The retention of the active agent within the polymer is determined by the chemical nature of the core (molecular weight, chemical functionality, polarity and volatility), polymer properties and the encapsulation technique. The solvation of the microcapsules, the density, the compressibility index and the angle of rest can also be determined (Patel et al. 2008).

Thus there are numerous interesting advantages, and therefore the microencapsulation technology is used for several purposes: to combine properties of different materials (e.g., organic and inorganic); to protect sensitive, unstable and reactive materials from their environments and prevent the degradation of active compounds (e.g. from reactions such as oxidation and dehydration) (Dubey et al. 2009) (Mishra et al. 2013); to protect the immediate environment of the microcapsules of the active components; to increase stability (Agnihotri et al. 2012); for controlled, delayed or sustained release; to reduce the dosing frequency; for immobilization of enzymes and microorganisms; to mask undesired properties of the active components (such as odor, taste and activity) (Dubey et al. 2009); for a targeted release of encapsulated materials ; for better processability, since it allows to improve the solubility, dispersibility and fluidity; for safe and convenient handling of toxic materials and for separating incompatible components for functional reasons.

2.2.1 Microencapsulation techniques

There are several hundred microencapsulation methods and no process is adaptable to all core materials or product applications. The difference between them is in the wrapping or entrapment of the material to be encapsulated by the encapsulating agent since the final product is a suspension of microparticles in which the size is variable. The choice of the most suitable method depends on the application of the microsystem, required particle size, physical and chemical properties of the core and polymer, desired release mechanism, production scale and costs. Ideally, the microencapsulation method should be simple, fast, reproducible and easy to transpose to the industrial scale and the major limitations of the methods are the high costs of all processing as well as the lack of availability of certain encapsulating materials. Each microencapsulation process depends on several aspects. However, the fundamental principle is common to all. In general, this corresponds to the deposition of the encapsulating agent on the agent to be encapsulated, following a series of steps. Initially, the encapsulating agent is

dissolved or molten, and in turn, the agent to be encapsulated may be present in the form of small or droplet particles or even in the form of gas. The material to be encapsulated is placed in an appropriate medium and thereafter the encapsulating agent is deposited thereon. Finally, the encapsulating agent undergoes solidification by forming the microparticles.

Microencapsulation techniques can be divided into two main categories, chemical and physical, the latter being subdivided into physical-chemical and physicomechanical techniques (Estevinho et al. 2013). Table 2 describes the common methods used to encapsulate active compounds and the size of the particles they produce.

Table 2 - Methods used in microencapsulation and the respective particle size produced. (Adapted from (Brasileiro 2011)*)*

Classification	Technique	Particle Size (μm)
Chemical	Polymerization	1-1000
Physical-chemical	Coacervation	1-1000
	Solvent evaporation	0.5-1000
	Sol-gel encapsulation	2-20
	Layer-by-layer assembly	0.5-20
	Supercritical fluid-assisted microencapsulation	0.5-500
Physical-mechanical	Spray-Drying	1-500
	Spray-cooling	20-500
	Polymer precipitation	5-1000
	Co-extrusion	250-2500
	Spinning disk	5-1500
	Fluidized-bed coating	20-1500
	Melt solidification	5-1000
	Polymer precipitation	5-1000

In pharmaceutical industry, microencapsulation by solvent evaporation is the most used method to obtain microparticles for DDSs, in order to achieve a sustained release of APC with a specific release profile. There are different techniques available for microencapsulation by solvent evaporation. An important factor to consider in the choise of the technique is the hydrophobicity or hydrophilicity of the APC. Double emulsion is a unique process that has the advantage of encapsulating both lipophilic and hydrophilic drug molecules. Both emulsion diffusion and coacervation techniques are used for incorporation of thermosensitive drugs whereas phase inversion temperature method cannot be utilized to encapsulate thermolabile actives like peptides and proteins. Lastly, the techniques that do not require the use of toxic solvents or organic solvents are: emulsion diffusion, microemulsion, nanoprecipitation, high

pressure homogenization and phase inversion temperature technique (Iqbal et al. 2015). Some prominent advantages and limitations of various techniques for encapsulation of drug are given in Table 3.

Table 3 - Advantages and limitations of various techniques used for encapsulation of drug

Techniques	Advantages	Disadvantages	Examples
Double emulsion solvent evaporation	- It provides an advantage of encapsulation of both hydrophilic and hydrophobic actives.	- Large and non-uniform particles (polydisperse). - Two step process. - Leakage of the hydrophilic active into external aqueous phase. - Difficult to scale up.	Bitar et al. 2015
Single emulsion solvent evaporation	- Provides high entrapment of lipophilic actives. - Size of particles is adjustable by changing homogenization speed, amount of stabilizer, viscosity of organic and aqueous phases.	- Entrapment of hydrophilic drugs is poor. - It is difficult to scale up.	Khalil et al. 2013
Emulsion diffusion method	- It allows incorporation of thermosensitive drugs. - Good batch–batch reproducibility. - Higher entrapment of lipophilic drugs. - Use of nontoxic solvents. - Easy scale up process	- Concentration of final formulation is required. - Possible organic solvent residues in the final formulation. - Poor encapsulation of hydrophilic drugs. - Longer time of emulsion agitation required.	Souguir et al. 2013
Microemulsion technique	- Reduces mean particle size and narrow size distribution. - Organic solvent free method. - No energy consuming process. - Easy to scale up.	- High concentration of surfactants and co-surfactants. - Concentration of final formulation is required	Destrée et al. 2007

Table 3 - Advantages and limitations of various techniques used for encapsulation of drug (cont.)

Coacervation method	- Allows incorporation of thermosensitive drugs. - Inexpensive for laboratory and industrial application. - Possibility to control shape and size of SLNs by reaction conditions.	- Possible degradation of the components under acidic conditions.	Wieland-Berghausen et al. 2002
Emulsion polymerization	- It is fast and scalable.	- Toxic organic solvents and monomers are used. - Difficult to remove residual monomers, initiators and surfactants from final product.	Tolue et al. 2009

SLNs - Solid lipid microparticles

2.2.1.1 Double emulsion by solvent evaporation technique

The first bibliographic reference to DEs, also known as emulsions of emulsions, is made by Seifriz, in 1924, but more detailed and intensive research on this technique was started at the end of 1970s. This method was initially described in review articles by (Florence & Whitehill 1981; Florence & Whitehill 1982), (Matsumoto et al. 1980) and (Frenkel et al. 1983). Solvent evaporation is a simple method frequently used since it allows the encapsulation of hydrophobic and hydrophilic substances. In this method, the polymer is dissolved in an immiscible water solvent and the encapsulated substance is dispersed or dissolved in the mixture.

The double emulsions may be the water-in-oil type ($w_1/o/w_2$) (with dispersed oil globules containing smaller aqueous droplets) or oil-in-water type ($o_1/w/o_2$) (with dispersed aqueous droplets containing small droplets dispersed oils). In fact, double emulsions present many interesting possibilities for the controlled release of chemicals initially included in the household. In this technique, $w_1/o/w_2$, the aqueous solution in which the drug is dissolved is emulsified in an organic phase containing (lipophilic emulsifier) the polymer, giving the first emulsion. This is then dispersed in a second aqueous phase (hydrophilic emulsifier), forming the second emulsion. After evaporation of the volatile solvent, the microparticles are collected. In Figure 4 the process used for the formation of microparticles by the method of evaporation of the solvent in water/oil/water double emulsion is schematized.

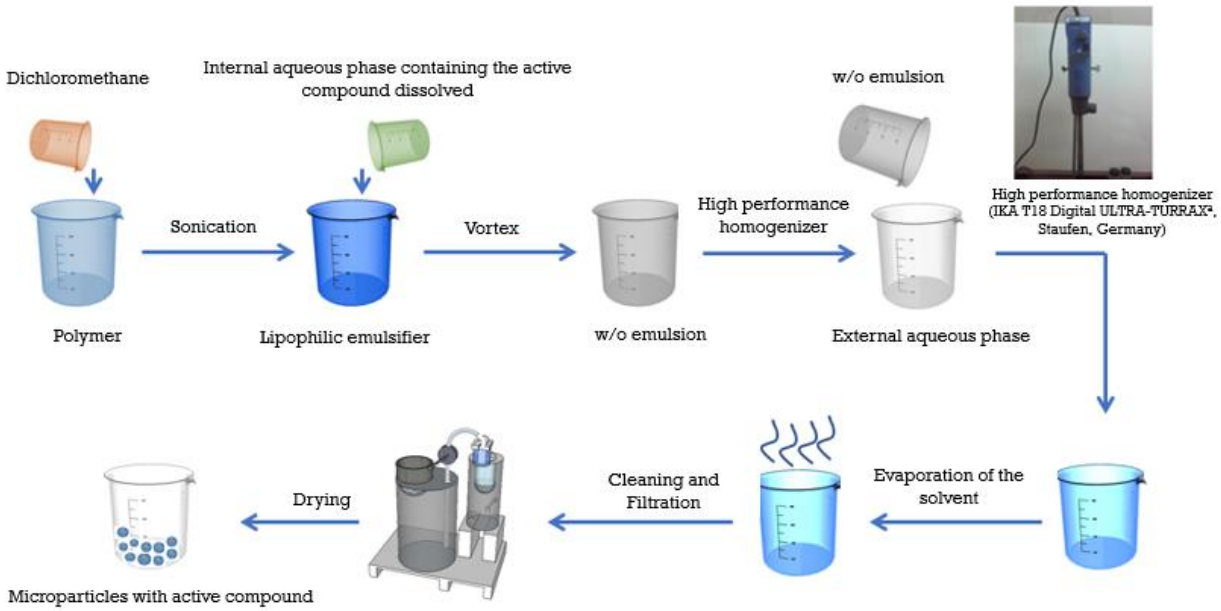


Figure 4 - Schematic representation of double emulsion by solvent evaporation

Several factors affect the formation of microparticles which should be thus optimized. The rate of solvent evaporation, the polymer molecular weight, the agitation rate, the organic

phase volume are some examples of parameters than can interfere with the microparticle formation.

The active substances may also migrate from the outer phase to the inner phase of multiple emulsions, thereby providing a type of reservoir particularly suitable for detoxification (overdose treatment) or, in a different domain, for the removal of toxic materials from the waste water. In any case, the impact of dual emulsions designed as drug delivery systems would be of significant importance in the field of controlled release provided that such stability and control mechanisms are clearly understood and monitored.

In s/o/w technique, the internal solid phase and external aqueous phase are separated by an oil layer. Firstly the solid pharmaceuticals or biopharmaceuticals are dispersed in the polymer solution to form a primary emulsion. Then the dispersion is introduced into a large volume of aqueous solution containing emulsifying agent, such as PVA or PEG (Giri et al. 2013). The s/o/w technique possesses two major advantages compared to $w_1/o/w_2$ technique: the first, s/o/w technique need not prepare w/o primary emulsion, whose stability is a prerequisite for the successful stabilization of a multiple emulsion and the high loading of drug within the solid microparticles; the second, the drug in solid state requires a dissolution step prior to the diffusion into continuous phase, thus allowing higher encapsulation efficiency (Wang et al. 2008). However, this technique requires a very low drug particle size so that allows a complete encapsulation of the drug crystals. Moreover, other drawbacks of the s/o/w technique might be the tendency of the drug to show sedimentation or flotation (caused by adhesion of gas bubbles to the hydrophobic surface due to low wettability) during the encapsulation process and, in the later stages of the product development, difficulties can also be expected during scaling up to large-scale manufacture (Wischke & Schwendeman 2008).

2.2.2 Microencapsulation in the pharmaceutical industry

The first research related to microencapsulation of pharmaceutical compounds was published in 1931 by Bungen Burg of Jon and Kan, who obtained gelatine microparticles through the coacervation process. Currently, the research on microencapsulation for pharmacological compounds is focused on the discovery of new drug delivery systems to obtain products to reach the market, reducing adverse reactions and side effects, being adequate to mask the bitter taste of various drugs, reduce irritations in the gastrointestinal tract as well as the odor and volatility of various substances, separate incompatible substances, provide protection to substances to encapsulate against atmospheric effects, increasing shelf life and allowing a possible controlled and sustained release of compounds (Agnihotri et al. 2012). Thus, microencapsulation presents itself as a potential technological strategy to achieve the above objectives. An efficient drug delivery system is one that allows the active compound to reach the target site, in the time required and for the desired time, and for that, four main factors

are considered: route of administration, active compound release pattern, method delivery and production process also known as the formulation process (Sinha & Trehan 2003). When the active compounds are not microencapsulated and are given repeatedly, it makes the regimen more frequent and always under medical supervision and as such, microencapsulation emerges as a potential drug delivery strategy to overcome multiple issues associated with multiple administrations. The formulated microparticles must be biocompatible, stable, safe and demonstrate predictable degradation kinetics. However, other factors, such as chemical modifications at the surface of the particle, can optimize the system and thus be possible to use microencapsulation for drug delivery systems. However, there are few microencapsulated pharmaceuticals available on the market (Stevenson 2009). This can be explained in terms of size control and size distribution is difficult, resulting in reduced reproducibility of the production process, especially on a large scale. Thus, even with certain difficulties encountered in the implementation of microencapsulation for drug delivery systems, traditional therapeutic techniques have been progressively replaced by more advanced technologies, such as microencapsulation.

2.2.3 Encapsulating agents

In the microencapsulation process a very large number of encapsulating agents have been used, responsible for the coating of the active compounds, forming the microparticle.

The selection of the encapsulation method and wall materials is interdependent, for example, in the double emulsion by solvent evaporation technique, $w_1/o/w_2$, the encapsulating agent used must be soluble in organic solvents. The chemical and physical properties of the microparticles are also determined by the selected coating material. A key factor in the preparation of such systems is the choice of appropriate biodegradable polymer. The coating agent should be compatible with the core material, microparticles final destination and release mechanism; be able to form a cohesive film with the core material; allow stabilization of the core material; provide specific coating properties (stability, strength, flexibility); be inert towards active ingredients and allow controlled release under certain conditions. Other restrictions might include its availability and competitive price (Agnihotri et al. 2012), (Sris & Prabha 2012). The main parameters that affect biocompatibility and degradation rates are the material chemistry, its molecular weight, solubility, shape and structure of the polymer, hydrophilicity/hydrophobicity, lubricity, surface energy, water absorption, degradation and erosion mechanisms.

The encapsulating agents may have different origins, from natural, semisynthetic or synthetic (Table 4).

Table 4 - Examples of encapsulating agents used in microencapsulation according to their origin (Brasileiro 2011)

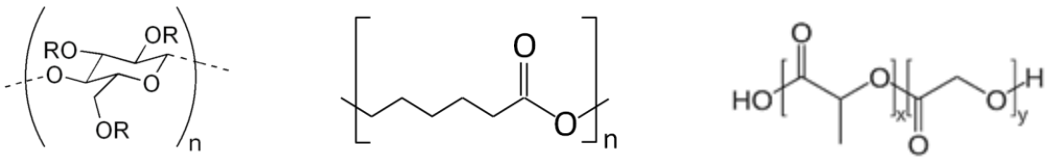
Types of Encapsulating Agents	Examples
Natural	Calcium alginate, sodium alginate, gum agar-agar, gelatine, chitosan, sucrose, dextran, caseinate and wax
Semisynthetic	Cellulose acetate, cellulose nitrate, ethylcellulose, methylcellulose, hydroxypropylcellulose, sodium carboxymethylcellulose, myristyl alcohol, glyceryl mono-, mono, glycerol di-tristearate
Synthetics	PCL, PLA, PLGA

PCL – Polycaprolactone; PLA - Polylactic acid; PLGA – Poly(lactic-co-glycolic acid)

There are features for which an encapsulating agent can be considered ideal of which low viscosity at high concentrations; be easy to handle during the microencapsulation process; present low hygroscopic to prevent agglomeration and to aid its manipulation; have a good ability to incorporate the material to be encapsulated to prevent its loss; protect the material to be encapsulated from adverse circumstances; not reactive with the compound to be analysed; when administered orally, have a pleasant taste; be economical and lack aroma. In addition to these aspects, the encapsulating agent must have the ability to form a film cohesive with the core material, providing strength, impermeability and stability in the preparation.

For the microencapsulation of pharmaceutical compounds, there are several encapsulating agents. The most commonly used polymers to produce particles include polylactic acid (PLA), polyglycolide (PGA), poly(lactic-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 & Verma 2011). Moreover, PACA polymers were reported in medical sector and polyamidoamine (PAMAM) polymers were reported for bio-applications (Ammala 2013).

However, EC is one of the most used because of its low cost allied to the fact that it has been approved by the American Food and Drug Administration (FDA) for therapeutic applications (Rosa et al. 2012). Figure 5 shows the chemical structure of the polymers used in this study.



A B C

Figure 5 - Chemical structure of ethylcellulose (A), Polycaprolactone (B) and Poly (lactide-co-glycolide acid) (C) (Adapted from (Vueba 2006)*)*

Ethylcellulose is a derivative of cellulose in which some of the hydroxyl groups on the repeating glucose units could be converted into ethyl ether groups. It is a non-biodegradable, but biocompatible, tasteless, odourless, non-irritating and non-toxic cellulose-derived polymer. This polymer can be obtained from the etherification of ethyl chloride and is characterised by its low flammability. In addition, this compound is lightweight, oxygenated, heat stable and resistant to mechanical stress. The EC polymer is soluble in organic solvents such as ketones, alcohols, esters and ethers. Accordingly, EC is attractive for microencapsulation purposes as a coating material, since it protects the drug against the gastrointestinal tract (Stulzer & Silva 2007) or it may be administered intraduodenal to prolong intestinal absorption (Takishima et al. 2002).

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 (Dubey et al. 2009).

2.2.4 Controlled release of active pharmaceutical ingredients from microparticles

Controlled release is one of the main advantages of microencapsulation. The term controlled release can 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 (e.g. pH, temperature, moisture, enzymes, etc.) at a recommended rate, a desired place and a certain time (López-Córdoba et al. 2014). This is a crucial 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. 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 (Fredenberg et al. 2011).

There are several release mechanisms by which core material can be released: hydrolysis, diffusion through water-filled pores, osmotic pumping, erosion, water absorption/swelling etc (Dubey et al. 2009; Fredenberg et al. 2011). However, they can be summarized in four main processes that are presented in Figure 6.

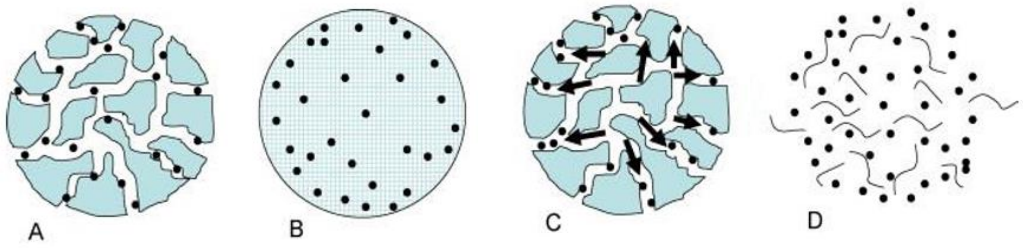


Figure 6 - Release mechanisms: (A) diffusion through water-filled pores, (B) diffusion through the polymer, (C) osmotic pumping and (D) erosion

Diffusion processes and osmotic pumping involve the transport of the core material whereas erosion is caused by polymer disintegration. Diffusion-controlled systems may be divided in reservoir or matrix types. Therefore, the release rate is influenced by the chemical properties of the core and coating material as well as the physical properties of the coating (e. g. pore size). In the matrix system, the core is homogenously scattered in the coating material, so the release rate depends on the core diffusion rate through the coating. Osmosis can be defined as the transport of core material through water-filled pore by a force such osmotic pressure (forced mass convection). Erosion is simply the disintegration of the polymer without any transport of the core material. Although it is possible to know the mechanism by which a substance could be released, it is difficult to assess which one is dominant and in a chain of processes, it may not be clear which one is the rate determining process (Fredenberg et al. 2011).

Transport through water-filled pores is the most common way of release, as the encapsulated drug is usually a biopharmaceutical, such as a protein or a peptide, which are too large and too hydrophilic to be transported through the polymer phase.

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 7. Core material release profile is frequently bi-phasic or tri-phasic. In a traditional tri-phasic release, phase I is 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. Phase II is characterized by a slow release, where

the core material diffuses through the pores or the polymer, while degradation or hydration of the shell material occurs. Phase III is usually described as a faster release profile (second burst) due to hydration or degradation and erosion of the polymer. According to Yeo & 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 hydrophobicity 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.

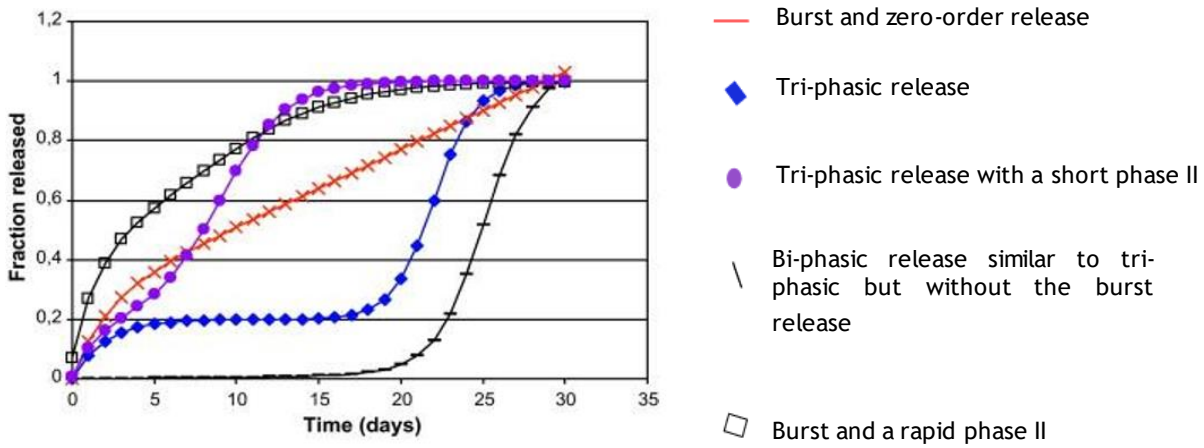


Figure 7 - Releases profiles consisting of different phases (Adapted from Fredenberg et al. 2011*)*

3 State of the art

In this section, published works on the microencapsulation of acetylsalicylic acid with different microencapsulating agents and methods will be presented and discussed. Table 5 summarizes the microencapsulating agents and techniques reported for acetylsalicylic acid, as well as some obtained results.

High doses of acetylsalicylic acid can be given to reduce inflammation, mean doses of acetylsalicylic acid can relieve pain, and administering low doses of acetylsalicylic acid can prevent blood clots caused by platelet aggregation, thus preventing cardiovascular disease caused by the thrombus (Shi et al. 2014). However, despite these attributes, acetylsalicylic acid has several side effects such as gastric irritation and bleeding, and studies have shown that the incidence of these gastrointestinal side effects may increase with regular use (Gugu et al. 2015). Therefore, a suitable dose should be used to reduce the adverse reaction of the gastrointestinal tract to acetylsalicylic acid. Previous reports have shown that the test compound in microencapsulated form is better absorbed, provided a sustained stable concentration of salicylates in plasma, produced significantly fewer gastric ulcerations and were much more tolerated compared to crude or conventional acetylsalicylic acid. The use of microcapsules to achieve various goals, such as environmental protection, increased stability, sustained or controlled release, is well established, and acetylsalicylic acid was one of the first candidates for microencapsulation.

Gugu et al. 2015 were developed a lipid based delivery system for acetylsalicylic acid and evaluate its physicochemical and pharmacodynamic properties. For this, they formulated solid lipid microparticles (SLMs) loaded with acetylsalicylic acid by the hot homogenization technique. The results suggested that the microparticles were spherical and smooth through analysis to the particle size and morphology. In addition, the authors stated that particle size is not directly proportional to loading and that encapsulation efficiency varies directly with particle size and inversely with loading. However, the main conclusion was that the formulation can be used for twice daily application because an initial high concentration is achieved, i.e., above the minimum effective concentration, before maintaining the dose over an extended period and therefore it is necessary to ensure that acetylsalicylic acid SLMs will come on the market soon so that patients can benefit from them.

According to Shi et al. 2014, the drug-polymer delivery system of acetylsalicylic acid /CS-NPs was exhibits well sustained release performance. In this study, they studied the encapsulation of acetylsalicylic acid with chitosan, namely the drug release properties, varying the molecular weight. By ionic gelation technology, they obtained spherical and smooth NPs,

but the particle size was not symmetrical in the distribution, with NPs being agglomerated. The results showed that the increase in the initial concentration of acetylsalicylic acid decreases the EE. Regarding *in vitro* drug release studies, it was concluded that it is possible to control the release rate of drug by adjusting the concentration of acetylsalicylic acid and molecular parameters of chitosan.

Moreover, according to Das et al. 2012, it was also possible to obtain acetylsalicylic acid NPs with albumin for ophthalmologic applications by a coacervation method, i.e. they evaluated NPs for their suitability as ocular carriers for the delivery of acetylsalicylic acid into the posterior chamber of the eye. The results were suggest the feasibility of using acetylsalicylic acid loaded albumin nanoparticles <200 nm in size with or without a coating of 0.5% xanthan gum, in the eye for treatment of diabetic retinopathy with better tolerance than the free drug. Further, *in vivo* studies was required to confirm the clinical relevance of these findings.

Another study, Liu et al. 2015, reports the preparation of acetylsalicylic acid microparticles loaded with PLGA-PEG-PLGA by the emulsion solvent evaporation technique and their release. In addition, a copolymer (Montmorillonite - MMT) was added and its influence on the release studies was studied. The results suggested that when the dose of drug encapsulated by the microparticles increases, the amount of drug release increases correspondingly. Their main finding was that the encapsulation of drugs using PLGA-PEG-PLGA/o-MMT microparticles can reform problems such as short drug half-life, excessive doses in the body and the frequency of drug delivery.

In the study of Dash et al. 2010, acetylsalicylic acid microcapsules were also formulated by the same method, emulsion solvent evaporation, but using as encapsulating agent ethylcellulose, cellulose acetate phthalate (CAP) and their mixtures (EC + CAP). The studies revealed that EC-based microcapsules were larger than CAP-based and EC + CAP-based microcapsules and the higher drug entrapment in CAP microcapsules was attributed to the percentage yield, nature and concentration of polymer in the internal phase. The results indicated that EC and CAP combination based formulation exhibited the slowest release rate in simulated gastric fluid (SGF) followed by a faster release in simulated intestinal fluid (SIF). The main conclusion from this study was that acetylsalicylic acid microcapsules could be made suitable for oral controlled drug delivery systems using cellulose acetate phthalate and ethyl cellulose as retardant materials.

Thanoo et al. 1993b, using the same technique, solvent evaporation technique, formulated polycarbonate microspheres containing high drug payloads, which can float in gastric and intestinal fluids to administer drugs such as acetylsalicylic acid and griseofulvin. The results showed that a slightly increased release rate was initially observed from smaller

particles compared to larger particles. In addition, as the acetylsalicylic acid release pattern in both simulated fluids was similar, oral administration of these microspheres did not affect the release profile, whereas the poor water-soluble drugs, p-nitroaniline and griseofulvin showed slower release. Thus, the main conclusion was that polycarbonate as a matrix may be more favourable for the controlled release of drugs with moderate water solubility, such as acetylsalicylic acid and p-nitroaniline.

Yang et al. 2000, formulated acetylsalicylic acid microcapsules with the EC encapsulating agent, using an oil-in-water emulsification solvent evaporation technique. The results showed that a higher concentration of polymer provides better encapsulation resulting in a higher loading efficiency of acetylsalicylic acid and that an increase in dispersed phase viscosity facilitates the coalescence of dispersed emulsified droplets. However, the larger size and the smooth surface caused by a higher concentration of polymer reduced the rate of dissolution.

Similar studies show the effect of microencapsulated ASA on the inhibition of human serum glycosylation ((Juretić et al. 1990)) and on antiplatelet activity ((Brown et al. 1999), (Al-Gohary et al. 1989)) compared to the free drug. These authors showed that the microencapsulated drug was more effective than the free acetylsalicylic acid, being associated with less effects in the gastrointestinal tract.

Al-Gohary et al. 1989 also microencapsulated acetylsalicylic acid with Eudragit by the phase separation technique to evaluate the antithrombotic effect. The results showed that Eudragit RL and RS are polymeric materials suitable for the preparation of slow release acetylsalicylic acid tablets with similar properties; the tablets produced are more stable under high temperature and humidity conditions compared to acetylsalicylic acid simple tablets and related storage alterations in the disintegration and release of the drug are in agreement and show that the film resistance to drug release increases with storage having an antithrombotic effect

In conclusion, microcapsules have been used as drug delivery systems in the pharmaceutical field for sustained or controlled release of drugs, and for artificial cells and organs. Biodegradable polymers have been widely used in this field. In addition, there are currently no studies of acetylsalicylic acid microencapsulation, in order to control its adverse effects on the gastrointestinal tract, and therefore, this study is advantageous.

Table 5- Studies on microencapsulation of the active pharmaceutical compound: Acetylsalicylic Acid

Method	Objectives	Encapsulating material	Results	Reference
Solvent evaporation	To evaluate the effect of microencapsulated acetylsalicylic acid on glycosylation of serum proteins *in vitro* in comparison with the free drug.	Poly(lactic acid)	- The microcapsules obtained were of roughly spherical shape - Capsules ranging in diameter from about 20 to 230 pm - Drug content of 16.6%	Juretić et al. 1990
Solvent evaporation	Encapsulating acetylsalicylic acid in ethyl cellulose microcapsules by solvent evaporation in an O/W emulsion	Ethylcellulose	- Through the addition of non-solvent in the dispersed phase, ethylcellulose deposition on the reactor wall has been alleviated - The recovered total weight increases with an increase in the polymer concentration - Larger microcapsules have a lower dissolution rate, resulting from the smaller total surface area - The dissolution rate increases with an increase in the amount of non-solvent, as a consequence of having a coarser surface and larger pores.	Yang et al. 2000
The phase separation	To study the effect of storage at relatively high temperature and humidity of these tablets and to compare with the results obtained simultaneously for plain acetylsalicylic acid tablets.	Eudragit	- Eudragit RL and RS are suitable polymeric materials for the preparation of slow release aspirin tablets with similar properties - The storage-related changes in disintegration and drug release are in agreement and show that film resistance to drug release increases by storage	Al-Gohary et al. 1989
Coacervation	To evaluate of aspirin loaded albumin nanoparticles for their suitability as ocular	Albumin	- Particle size less than 200 nm in diameter - Drug release is much higher than 1-2 % - 81 % drug entrapment	Das et al. 2012

Table 5 - Studies on microencapsulation of the active pharmaceutical compound: Acetylsalicylic Acid (Cont.)

Emulsion solvent evaporation	To study the microencapsulation of acetylsalicylic acid and the study of its release kinetics.	Ethylcellulose, cellulose acetate phthalate (CAP), mixture (1:1) of polymeric constituents.	- Microcapsules spherical, free flowing and white in colour - The drug content was found to be higher in CAP microcapsules followed by EC and CAP+EC - The yield of microcapsules was 90–94 % - Drug release from all the microcapsules followed first order kinetics - EC and CAP combination based formulation exhibited the slowest release rate in SGF followed by faster release in SIF	Dash et al. 2010
Hot homogenization	To develop a lipid based delivery system for acetylsalicylic acid and to evaluate its physicochemical and pharmacodynamic properties.	Lipid matrix	- Batches A1 and B1 containing 1% of acetylsalicylic acid recorded the highest EE of 70 and 72%, respectively - EE varied directly with particle size and inversely with drug loading - The results show that maximum releases of 95.1 and 93.2% were obtained at 8 h from batches A1 (1% aspirin; Poloxamer) and B1 (1% aspirin; Soluplus), respectively	Gugu et al. 2015
Ionic gelation	The acetylsalicylic acid was encapsulated with different grades of CS varying in molecular weight (Mw) for the purpose of controlled release.	Chitosan	- NPs were spherical in shape with a smooth surface - The EE – 37% to 90% was significantly affected by the TPP concentration - The drug loading increased with increasing TPP concentration - Increasing the initial concentration will decrease the EE of acetylsalicylic acid - It is possible to control the release rate of acetylsalicylic acid by adjusting the concentration of acetylsalicylic acid	Shi et al. 2014

Table 5 - Studies on microencapsulation of the active pharmaceutical compound: Acetylsalicylic Acid (Cont.)

Emulsion solvent evaporation	Explore how the microparticles fabricated by encapsulating acetylsalicylic acid using PLGA-PEG-PLGA affect a localized drug delivery system	PLGA-PEG-PLGA	- SEM analysis indicated that the added concentration of PVA influenced the microparticle formation - When the drug dose encapsulated by the microparticles increased, the drug release amount increased correspondingly - The acetylsalicylic acid -loaded PLGA-PEG-PLGA steadily increased drug release within 20 h of reaction time and attained equilibrium in drug release after 50 h	Liu et al. 2015
Solvent evaporation	Prepare polycarbonate microspheres containing high drug payloads, which can float on gastric and intestinal fluids for delivering drugs such as aspirin and griseofulvin.	Polycarbonate	- The microspheres obtained were spherical - The release rate of acetylsalicylic acid is several times faster than either p-nitroaniline or griseofulvin - The formulation having 60% acetylsalicylic acid produced a larger proportion of bigger particles - The drug incorporation efficiency in the case of acetylsalicylic acid was found to be high if the initial loadings were high	Thanoo et al. 1993b
Carrying out the cross-linking reaction	Prepare of cross-linked PVA microspheres containing drugs and evaluate the release of the drugs into simulated gastric and intestinal fluids in-vitro.	Glutaraldehyde	- Good spherical geometry - EE = 93 % - Acetylsalicylic acid showed moderate release rate and the rate is slightly faster in intestinal fluid than in gastric fluid - The amount of drug initially present in the microspheres did not influence the rate of release to any significant extent - The rate of release of the drugs was found to be considerably influenced by the cross-linking density	Thanoo et al. 1993a

CAP - Cellulose acetate phthalate; CS – Chitosan; EC – Ethylcellulose; EE - Efficiency encapsulation; NPs – Nanoparticles; PEG - Polyethylene glycol; PLGA – Poly (lactic-co-glycolic acid); PVA - Poly (vinyl alcohol); SEM - Scanning electron microscope*; SGF - Simulated gastric fluid; SIF - Simulated intestinal fluid*; *TPP- Tripolyphosphate*

4 Materials and Methods

4.1 Materials

4.1.1 Reagents

Acetylsalicylic acid, ASA, with 99% purity (CAS 50-78-2) was purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Ethylcellulose, EC, (Ref. 433837-250G, viscosity of 46 cP, CAS 9004-57-3) and Polyvinyl alcohol, PVA, (Ref. P8136-250G, 87-90% hydrolyzed, average molecular weight de 30000-70000, CAS 9002-89-5) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Dichloromethane, DCM, (Ref. 1064541000, CAS 75-09-2) was obtained from (Merck, Darmstadt, Germany). Ethanol (Ref. 121085.1212, CH_3CH_2OH, 96% v/v PA, CAS 64-17-5) Panreac (Barcelona, Spain). Polycaprolactone, PCL, (Ref. 900288, CAS 24980-41-4) and Poly(D,L-lactide-co-glycolide), PLGA, (Ref. 739944-5G, CAS 26780-50-7) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Dimethyl sulfoxide, DMSO, (Ref. 506008, CAS 67-68-5), Ethyl Acetate, EA, (Ref. 205-500-4, CAS 141-78-6), Carboxymethylcellulose, CMC, (Ref. 419273-100G, CAS 9004-32-4) and Hydrochloric acid, HCl, (Ref. 231-595-7, CAS 7647-01-0) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). α-Amylase from porcine pancreas (Ref: A6255-10MG, ≥1000 units/mg protein), Bile Salts (Ref. 48305-50G-F), Pepsin from porcine gastric mucosa (Ref. P7000-25G, ≥250 units/mg solid) and Pancreatin from porcine pancreas (Ref. P3292-25G) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). The water used in this work was de-ionized and double-distilled using a MilliporeTM water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

4.1.2 Equipments

All weight measurements were performed using a Mettler Toldedo AG245 analytical balance (Columbus, OH, USA). The spectrophotometer UV-Vis V-530 (Jasco, OK, USA) was used in the validation of analytical methods and to obtain the encapsulation efficiency. The preparation of the ASA 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) was 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). Particle size distribution was measured by laser granulometry using a

Coulter Counter-LS 230 Particle Size Analyser (Miami, FL, USA). pH measurements were performed using a 900 Multiparameter Water Quality Meter (A & E Lab; Guangzhou, China). The samples were sputter-coated with gold for 20 seconds using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany). A PHENOM XL scanning light microscope (Eindhoven, The Netherlands) at an accelerating voltage of 10 kV was used to evaluate the acetylsalicylic acid - loaded EC/PLGA/PCL microparticles external morphology and polydispersity. Freeze-dried microparticles were placed on an aluminum stub with a carbon double-sided adhesive tape. For the controlled release studies of acetylsalicylic acid *in vitro* simulated gastrointestinal fluids, a Lovibond incubator (Amesbury, United Kingdom) at 37 °C, a horizontal shaker (Orbital IKA KS 130 basic, Germany) at 170 rpm and a 0.2 µm syringe filter (Ref: 514-0070, VWR International, Fontenay-sous-Bois, France) were used.

4.2 Methods

4.2.1 Analytical methods validation

Analytical preparation of the stocks solutions and respective standards in the different simulations

A stock solution of 4.00 ± 0.10 $g.L^{-1}$ (1) of acetylsalicylic acid was prepared in ultrapure water for the method $w_1/o/w_2$, accurately measuring 400.00 ± 0.01 mg ASA, using an analytical balance (Mettler Toldedo) in a volumetric flask of 100.00 ± 0.10 mL. The stock solution was sonicated for 1 hour and 15 minutes, then sealed with parafilm, wrapped in an aluminum foil to protect from light and stabilized for 18 hours at room temperature to ensure homogenization. An intermediate solution (2) (for validation) of 2.00 ± 0.10 $g.L^{-1}$ of acetylsalicylic acid was also prepared solution from the stock solution by dilution in ultrapure water in a 25 mL volumetric flask. The intermediate solution was stabilized for 18 hours at room temperature before the standards were prepared. The ultrapure water was filtered through 0.45 µm Nylon 66 filter membranes (VWR) and adjusted to pH with 0.1175 M of HCl. This pH was adjusted to pH 2 so that it was lower than pKa of the pharmacologically active compound in question, because if it was higher, it would go into its deprotonated form (Figure 8). For the simulation of the salivary, gastric and intestinal fluids, the same concentrations of standard solutions were prepared, and the intermediate solution was diluted in the respective simulated fluids.

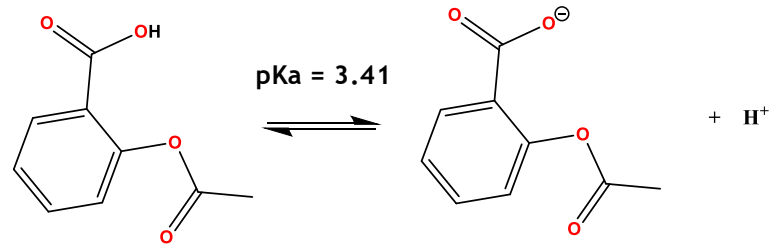


Figure 8 – The chemical structure of acetylsalicylic acid in its protonated and deprotonated form

Other stock solutions were prepared for the s/o/w method. Firstly, a stock solution of 64.00 ± 0.10 mg.L^{-1} (4) of acetylsalicylic acid was prepared in ethanol, accurately measuring 3.20 ± 0.01 mg ASA, using the same equipment, in a volumetric flask of 50.00 ± 0.10 mL. Another stock solution of 56.00 ± 0.10 mg.L^{-1} (5) of acetylsalicylic acid was prepared in PVA, accurately measuring 2.80 ± 0.01 mg ASA, using the same analytical balance, in the same a volumetric flask. For each of these solutions, (4) and (5), a intermediate solution of 50.00 ± 0.10 mg.L^{-1} of ASA was prepared from the respective stock solution by dilution in ethanol and PVA, respectively, in a 25 mL volumetric flask. Finally, a stock solution of 72.00 ± 0.10 mg.L^{-1} of acetylsalicylic acid (7) was prepared in acidified PVA, accurately measuring 3.60 ± 0.01 mg ASA, using the same equipment, in a volumetric flask of 50.00 ± 0.10 mL. All these solutions were sonicated and stored under the same supra-referenced conditions.

All the working standard solutions of ASA also in a range of pre-defined concentrations, in 10.000 mL ± 0.025 mL volumetric flasks, were prepared by dilution of the intermediate solution or stock solution in the respective fluid. The standard solutions were stabilized for 2 hours at room temperature under the same supra-referenced storage conditions, prior to analysis (Table 6).

UV-Vis Spectrophotometry

All spectrophotometric analyses were performed using a Jasco V-530 UV-Vis spectrophotometer with quartz cells of 10 mm of light path. The SPECTA MANAGER software was used for all the absorbance measurements. To determine the detection wavelength for analysis, in which the maximum absorption occurred, one intermediate solution of ASA in all the mediums investigated, except the simulated gastrointestinal fluids, were scanned between wavelengths of 190 to 900 nm and the maximum absorption spectra were obtained. Considering the maximum absorption wavelength from the spectra, detection and quantification of ASA was performed, in all spectrophotometry analyses using ultrapure water pH 2, PVA, ethanol and acidified PVA. For the simulated fluids, 3 solutions were prepared, where 50 µL of the

intermediate solution was withdrawn and completed with the respective medium and finally the wavelength was determined for the maximum absorption for each under the above conditions (Table 6). All the standards of ASA were analysed for all the mediums investigated, as described in Table 6, except for the $w_1/o/w_2$ emulsification method, which was only in the ultrapure water pH 2 and simulated fluids. Therefore, it was necessary to obtain a linear relation through the realization of calibration curves for all the different mediums used.

Table 6 – Summary of the concentrations used for the preparation of the standard solutions of ASA and the maximum absorption wavelength, for all the mediums investigated

Medium	Maximum absorption wavelength (nm)	Concentration Range (mg/L)
Ultrapure Water pH 2	247	2.0 - 20.0 mg/L
PVA	243	0.125 - 10.0 mg/L
Acidified PVA	347	0.125 - 30.0 mg/L
Ethanol	242	4.0 - 40.0 mg/L
Simulated gastrointestinal fluids	296	2.0 - 20.0 mg/L

PVA - Polyvinyl alcohol

Validation of calibration curves

The UV-Vis spectrophotometry method was used to validate the calibration curves and thus demonstrate that this method is suitable for the quantitative determination of acetylsalicylic acid and to guarantee the reliability of the results. For this, the quantification parameters (linearity, sensitivity and limits of detection and quantification) were determined.

Linearity is the ability of a method to demonstrate that the results obtained are directly proportional to the concentration of the analyte in the sample within a specific range (Skoog et al. 1992). The results of the analysed solutions were processed statistically to determine the equation of the calibration line and the coefficient of correlation R^2, using the software Microsoft Excel 2016. The calibration line was constructed, where the absorbances and concentrations of ASA in the vertical axis were represented in the vertical axis and horizontal axis. Linearity should be assessed from the analysis of at least 5 standard concentrations at a range factor greater than 10, and the correlation coefficient should be at least 0.95. The sensitivity of the method is expressed as the slope of the calibration line.

In order to validate the calibration curves and subsequently the analytical method the following conditions must be met (Skoog et al. 1992):

- Analysis of at least 5 different standard solutions concentrations;
- Linearity range in a factor superior to 10;

- $R^2 \geq 0.95$;
- $Sa/a \leq 5\%$;
- $b - sb < 0 < b + sb$.

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

The limit of detection (LOD) is the lowest amount of acetylsalicylic acid that can be detected in a sample while the limit of quantification (LOQ) is the lowest amount of acetylsalicylic acid that can be quantified with acceptable accuracy and precision (Forootan et al. 2017). In UV spectrophotometry, LOD and LOQ can be calculated through the following expressions:

$$LOD = \frac{3 \times s_b}{a} \quad \text{(Eq.1)}$$

$$LOQ = \frac{10 \times s_b}{a} \quad \text{(Eq.2)}$$

4.2.2 Preparation of the microparticles with acetylsalicylic acid (Microencapsulation)

In this project, as already mentioned, the double emulsion technique was used by solvent evaporation, with three different types of emulsions, and each one with several formulations, varying some parameters (Table 7). All sets were performed in triplicates.

Table 7 - Summary of the differents formulations of ASA microparticles performed for this project

Technique	Formulation number	Formulation parameter
s/o/w	1	Solid ASA; PLGA; DCM; PVA 1%
	2	Solid ASA; EC; DCM; PVA 1%
	3	Solid ASA; PCL; DCM; Acidified PVA 1%
	4	Micronized solid ASA; PCL; DCM; Acidified PVA 1%
	5	Solid ASA; EC; EA; Acidified PVA 1%
$w_1/o/w_2$	1	Stock solution; EC; DCM; Acidified PVA 1%
	2	Stock solution; PCL; DCM; Acidified PVA 1%
	3	Stock solution; PLGA; DCM; Acidified PVA 1%

ASA – Acetylsalicylic acid; DCM - Dichloromethane; EA - Ethyl acetate; EC – Ethylcellulose; PCL – Polycaprolactone; PLGA - Poly(lactide-co-glycolide acid); PVA - Polyvinyl alcohol

Emulsification method - s/o/w

In this technique, the pharmacologically active compound is present in the solid state and approximately, 339.71 ± 0.01 mg ASA, were weighted using a spatula to a watch glass dish. This phase can be micronized with the aid of a mortar, to decrease particle size (depending on the intended formulation). This is referred to as a "Solid Phase".

Then, approximately 336.35 ± 0.01 mg of the encapsulating agent (EC; PLGA or PCL- depending on the intended formulation), was weighted on a calibrated analytical balance using a spatula to an ambar flask of 25 mL. The solvent used was dichloromethane, and 4.485 mL of this was 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 during 10 minutes or more, 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 acidified (can´t be acidified, depending on the intended formulation) of PVA 1% (w/w) was prepared by weighing 5.00 g ± 0.01 g of the polymer to a flask of 100 mL, using a spatula and a calibrated analytical balance, as well as by weighing 495.00 g ± 0.01 g of ultra-pure water to a glass bottle of 1000 mL. Afterwards, the flask with ultra-pure water was put inside a pan in an AREX Digital stirring plate to heat to a temperature of 95 °C (the PVA is not soluble at room temperature), 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 for to cool down and 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. Then, 0.1175 M of HCl was added dropwise with the aid of a pipette to pH 2. This solution was named "External or Continuous Aqueous Phases – W_2".

After the formation of the previous phases, the solid 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 (Figure 9) for 5 minutes at 5 000 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 fumehood 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 distilled water and filtered using a Vacuum Filtration System 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 24 hours. The freeze-drying studies were performed to determine the optimal time the microparticles need to be lyophilized using a bench top freeze-dryer (SP Scientific, NY, USA). After the filtration process, the microparticles were collected into vials. These microparticles were then lyophilised in those vials. After the first 24 hours of lyophilisation, all the vials were weighted and the absolute value of the weight of the difference between those two instances of time was calculated in order to determine the standard deviation and relative standard deviation.

Emulsification method - $w_1/o/w_2$

Firstly, a stock solution of ASA (4.00 g/L) was previously prepared. The volumetric flask was filled up with ultrapure water to the height of the meniscus using a pipette, and the sample was kept at the room temperature for 18 hours, for further stabilization. This final solution was named "Internal Aqueous Phase – W_1".

Afterwards, 100.00 ± 0.01 mg of the encapsulating agent (EC; PLGA or PCL- depending on the intended formulation), was weighted on a calibrated analytical balance using a spatula to an ambar flask of 25 mL. The solvent used was dichloromethane, and 10 mL, 1.429 mL and 1.333 mL of this were added to the previous polymer flask using a micropipette, for formulation 1, 2 and 3, respectively. The amount of solvent used was determined by the ratio of the polymer mass to the solubility thereof. 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 during 10 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".

The external phase is explained above.

After the formation of the previous phases, 1 mL of the internal aqueous phase was added to the organic phase in formulations with PCL and PLGA, but in the EC formulation, 5 mL of the internal aqueous was added to the organic phase. The following steps were the same as the s/o/w technique.

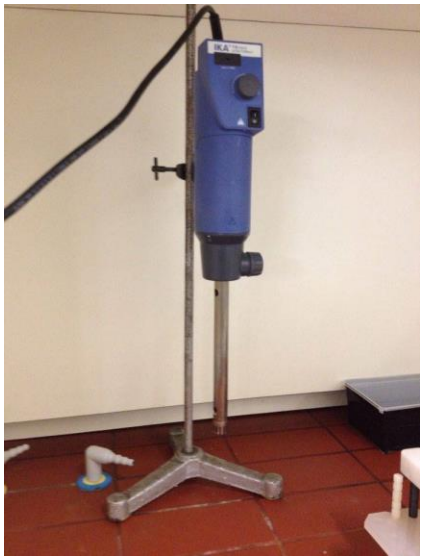


Figure 9- High-Performance Homogenizer (IKA T18 ULTRA-TURRAX®, Staufen, Germany)

4.2.3 Characterization of the microparticles

Microparticles were characterized according to their morphology and size distribution (Figure 10). Product yield, encapsulation efficiency and loading were also calculated using the equations below, for an average of 3 samples.

Product yield

Product yield was calculated for all the experiments as the ratio between the mass of the output powder obtained at the end of the freeze-drying process and the total initial mass, i.e., a mixture between the encapsulating agent and active compound (acetylsalicylic acid). PY was calculated according to the following equation:

$$\boldsymbol{PY}\ (\%) = \left(\frac{Weight\ of\ microparticles}{Weight\ of\ polymer + Weight\ of\ Acetylsalicylic\ Acid} \right) \times 100 \qquad \text{(Eq.3)}$$

Encapsulation efficiency

Encapsulation efficiency was measured for all the experiments as the ratio between the amount of core (acetylsalicylic acid) inside the microparticles and the total amount of active compound. To determine the amount of core trapped within the microparticles, the difference was made between the total amount of encapsulated acetylsalicylic acid and the concentration released. Therefore, the encapsulation efficiency was measured using the following equation:

$$\boldsymbol{EE}\,(\%) = \frac{Weight\ Acetylsalicylic\ Acid\ encapsulated}{Weight\ of\ Acetylsalicylic\ Acid} \times 100 \qquad \text{(Eq.4)}$$

This parameter was obtained through the absorbance measurement on UV-Vis spectrometer of the supernatant obtained after 3 mL of the microparticles solution has been filtrated using syringe filter (Fontenay-sous-Bois, France) with a 0.2 $\boldsymbol{\mu}$m to ensure that PVA residues were retained in the filter.

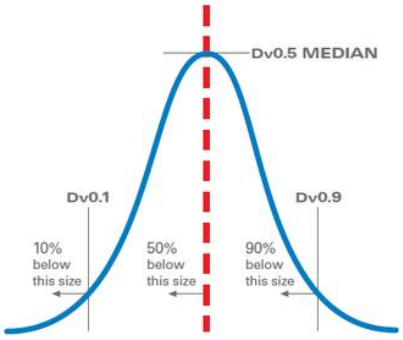
Loading

Loading (%) was determined by the ratio between the mass of ASA used and the mass of MPs obtained after lyophilization, i.e., corresponds to the amount of ASA that can be encapsulated in each microparticle.

$$\boldsymbol{Loading}\,(\%) = \frac{Weight\ of\ Acetylsalicylic\ Acid\ encapsulated}{Weight\ of\ microparticles} \times 100 \qquad \text{(Eq.5)}$$

Particle size distribution

The size distribution of the microparticles was evaluated by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA) equipped with small volume plus. For each experiment, a small sample of the powder was suspended in ultrapure water before measurement. The particles were characterized by volume distribution. The results were obtained as an average of three runs of 60 s. The value of SPAN (Eq.6) was calculated to evaluate the polydispersity of the system, showing the particle size distribution.



$$\boldsymbol{SPAN} = \frac{D_{v,90} - D_{v,10}}{D_{v,50}} < 4 \qquad \text{(Eq.6)}$$

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.

Particle morphology

A PHENOM XL scanning light microscope (Eindhoven, The Netherlands) at an accelerating voltage of 10 kV was used to evaluate the ASA-loaded EC/PCL/PLGA microparticles external morphology and polydispersity. Freeze-dried microparticles were placed on an aluminum stub with a carbon double-sided adhesive tape. The samples were sputter-coated with a goal for 20 seconds using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany).

4.2.4 Controlled release studies in the different simulations of the gastrointestinal tract

The *in vitro* release of the different formulations at different moments was evaluated in a simulated salivary fluid (SSF), in a simulated gastric fluid (SGF) and in a simulated intestinal fluid (SIF) with enzymes and bile salts in SIF. This three different mediums simulated were prepared, according to Table 8 and Table 9, and the pH was adjusted to 7 for SSF and SIF, and 3 for SGF. This step was performed according to (Minekus et al. 2014).

Table 8 – Composition of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

		SSF	SGF	SIF
Constituent	**Stock concentration (g/L)**	**Stock volume (mL)**	**Stock volume (mL)**	**Stock volume (mL)**
KCl	37.3	15.1	6.9	6.8
KH_2PO_4	68	3.7	0.9	0.8
$NaHCO_3$	84	6.8	12.5	42.5
NaCl	117	-	11.8	9.6
$MgCl_2(H_2O)_6$	30.5	0.5	0.4	1.1
$(NH_4)_2CO_3$	48	0.06	0.5	-

Table 9 – Enzymes used in all the mediums investigated

Oral Phase	Gastric Phase	Intestinal Phase
Salivary Amylase (75 U/mL)	Pepsin (2000 U/mL)	Pancreatin (100 U/mL) Bile (10 mM)

For the salivary simulation, 10 ± 0.01 mg of microparticles were weighted and added into a vial. Then 750 µL of ultra-pure water, 750 µL of SSF and 1.16 µL of α-amylase were added into the same vial. The vial was then vortexed for 30 seconds and put inside an incubator at 37 °C and on top of a horizontal shaker at 170 rpm. Samples were taken every 0, 1 and 2 minutes and filtered into another vial using a 0.2 µm syringe filter, in order to measure only the active ingredient.

For the gastric simulation, 10 ± 0.01 mg of microparticles were weighted and added into a vial. Then 750 µL of ultra-pure water, 750 µL of SGF and 6 mg of pepsin were added into the same vial. The vial was then vortexed for 30 seconds and put inside an incubator at 37 °C and on top of a horizontal shaker at 170 rpm. Samples were taken every 0, 30, 60, 90 and 120 minutes and filtered into another vial using a 0.2 µm syringe filter, in order to measure only the active ingredient.

For the intestinal simulation, 10 ± 0.01 mg of microparticles were weighted and added into a vial. Then 750 µL of ultra-pure water, 750 µL of SIF, 18.75 mg of pancreatin and 375 mg of bile salts were added into the same vial. The vial was then vortexed for 30 seconds and put inside an incubator at 37 °C and on top of a horizontal shaker at 170 rpm. Samples were taken every 0, 30, 60, 90 and 120 minutes and filtered into another vial using a 0.2 µm syringe filter, in order to measure only the active ingredient.

The amount released was evaluated considering the absorbance read of the sample using the respective simulated fluid as a blank.

Figure 10 - Methods used to characterize the microparticles obtained in this project

5 Results and Discussion

In this chapter the results regarding the analytical methods validation, acetylsalicylic acid microparticles characterization and controlled release studies in water and simulated fluids (SSF, SGF and SIF) will be presented, by the $w_1/o/w_2$ technique in three different formulations (using PCL, PLGA and EC). All the results obtained by the other technique will be neglected since they were not expected. The results are discussed in section 1 of this chapter. Subsequently, acetylsalicylic acid microparticles prepared by double emulsion solvent evaporation were characterized regarding its morphology, size, shape and loading. Encapsulation efficiency and product yield of the process are also present in section 2 of this chapter. Section 3 of this chapter reports the results regarding the controlled release studies of microparticles prepared. The release fluids were simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The release was performed for 2 minutes for the salivary fluid and 2 hours for the remaining fluids and UV-spectrophotometry analytical method was used to quantify the amount of acetylsalicylic acid released.

5.1 Analytical method validation

5.1.1 UV-Vis spectrophotometry

By UV-Vis spectrophotometry, the acetylsalicylic acid absorption spectrum was determined for the various simulations referred to above (saliva, stomach and intestine), ultrapure water at pH 2 and acidified PVA, to determine the absorption wavelength maximum for each portion of the gastrointestinal tract, water and for acidified PVA (Figure B1- Appendix B). In the simulated fluids (SSF, SGF and SIF) the results showed a maximum absorption at 296 nm, already in the case of ultrapure water at pH 2 the maximum absorption was at 247 nm. For the case of acidified PVA, the maximum absorption was at 347 nm.

The UV-Vis spectrophotometry method was used for the validation of the calibration curves in order to demonstrate that it is suitable for the quantitative determination of ASA and to guarantee the reliability of the results.

To obtain a linear relation, five sets of working standard solutions of acetylsalicylic acid in a range of pre-defined concentrations were analysed in five different mediums. These results allowed the construction of five calibration curves for the controlled release studies (Figure 11). The quantification parameters (linearity, sensitivity and limits of detection and quantification) were also determined.

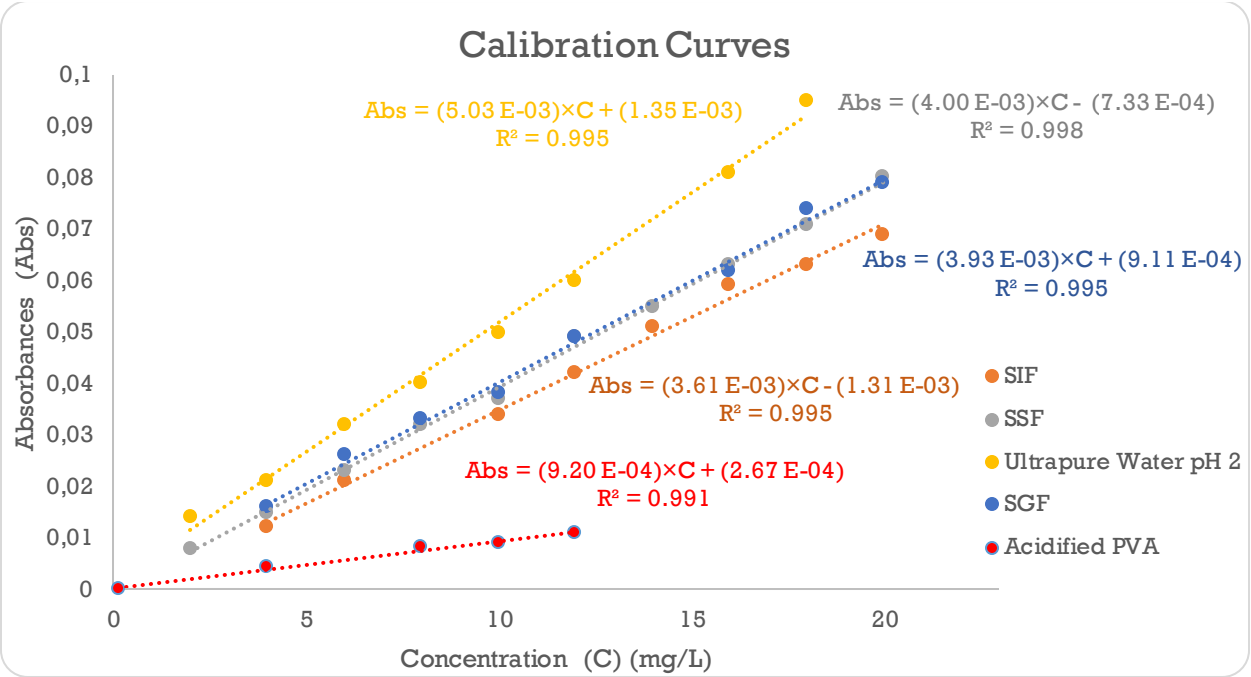


Figure 11 - Calibration curves of ASA for validation of the UV-Vis Spectrophotometry method in simulated fluids (SSF, SGF and SIF), UPW at pH 2 and acidified PVA

The parameters that allow the analytical method validation are listed in Table 10. All the five conditions for UV-Vis-spectrophotometry analytical method validation were verified, and so the analytical method was validated for all fluids.

Table 10- Linearity conditions for the validation of the UV-Vis-Spectrophotometry standard curves

Parameters	UPW	Acidified PVA	SSF	SGF	SIF
Number of standards concentrations	8	5	10	8	8
Linearity range (mg/L) ≥ 10	2-18	0.125-12	2-20	4-20	4-20
R^2 ≥ 0.990	0.995	0.991	0.998	0.995	0.995
Sa/a ≤ 5%	2.761	3.914	1.545	2.855	3.027
Intercept confidence interval (b-sb<0<b+sb)	-1.62×10^{-4} <0< 2.86×10^{3}	-1.36×10^{4} <0< 6.70×10^{4}	-1.50×10^{3} <0< 3.41×10^{5}	-5.43×10^{4} <0< 2.36×10^{3}	-2.79×10^{3} <0< 1.73×10^{4}
LOD	0.900	0.102	0.095	0.987	1.232
LOQ	3.000	4.377	0.126	1.113	2.454

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 controlled release studies, meaning that the concentrations used in the samples were sufficient to be measured and determined with a satisfactory degree of accuracy and precision. The minimum amount released of ASA from microparticles in SSF, SGF, and SIF was corresponding to the concentrations of 0.196 mg/L, 0.245 mg/L, and 2.764 mg/L in formulation 1, 1.238 mg/L, and 8.220 mg/L in formulation 2 and 1.377 mg/L, and 8.059 mg/L in formulation 3, respectively and the LOQ values of 0.126 mg/L, 1.113 mg/L, and 2.454 mg/L. Both the LOD and the LOQ values were satisfactorily low, which demonstrate the possibility of application of the proposed analytical methods to the quantification of acetylsalicylic acid associated with microencapsulation purposes by UV–Vis method.

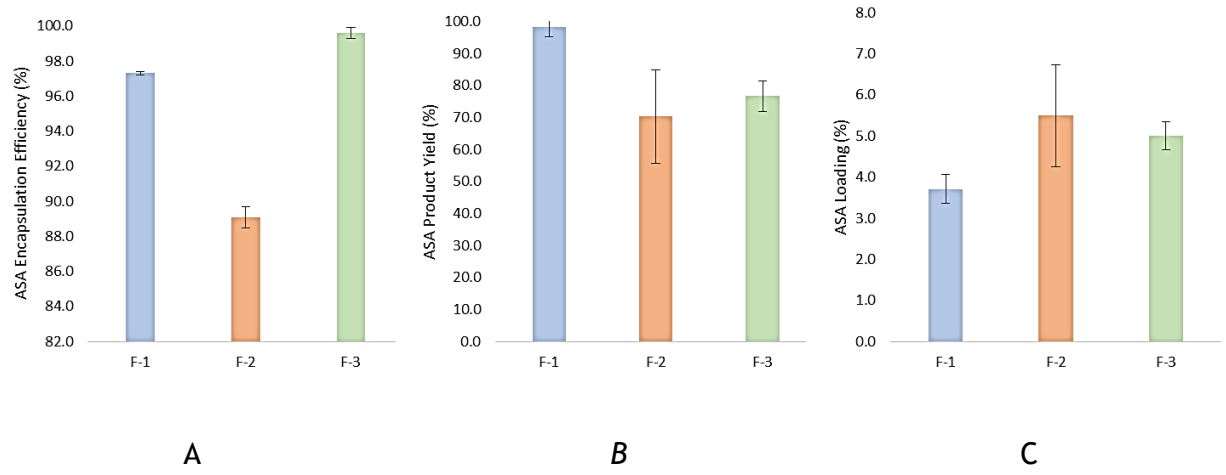
5.2 Microparticles characterization

Some characterization parameters of the microparticles were calculated. The results obtained for the EE, PY, and loading are described in Table 11 and Figure 12.

Table 11 – Microparticles characterization parameters obtained for the three formulations

	Formulation 1		Formulation 2		Formulation 3	
	Mean [a] (M ± s)	RSD (%)	Mean [a] (M ± s)	RSD (%)	Mean [a] (M ± s)	RSD (%)
Encapsulation Efficiency (%)	97.3 ± 0.1	0.1	89.1 ± 0.6	0.6	99.6 ± 0.3	0.3
Product Yield (%)	98.3 ± 3.0	3.1	70.3 ± 14.5	20.6	76.7 ± 4.8	6.2
Loading (%)	3.7 ± 0.4	9.6	5.5 ± 1.2	22.4	5.0 ± 0.3	6.7

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



A B C

Figure 12 - Results of the ASA encapsulation efficiency (A), product yield (B) and loading (C)

5.2.1 Product yield

Product yield is an important aspect of the encapsulation process since it reveals the quantity of powder that it possible to recover comparing to the initial raw materials used. The product yield was 98.3 ± 3.0% for formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%); 70.3 ± 14.5% for formulation 2 (Stock solution; PCL; DCM; Acidified PVA 1%) and 76.7 ± 4.8% for formulation 3 (Stock solution; PLGA; DCM; Acidified PVA 1%). The highest value was obtained for formulation 1 (5 mL of internal aqueous phase; acidified PVA 1%; EC; DCM as organic solvent), while the lowest value was obtained for formulation 2 (1 mL of internal aqueous phase; acidified PVA 1%; PCL; DCM as organic solvent). The achievement of these results may be due to the amount of the internal aqueous phase used. Thus, the greater the amount w_1 used, the greater the YP, because the amount of the pharmacologically active compound is also higher. The obtained results were satisfactory for the technique and the scale that was used and showed that there were no significant losses in the encapsulating process. It should be noted that microparticles could still contain remains of emulsifier or solvents if they were not properly washed and dried, resulting in higher product yield values.

A study that formulated acetylsalicylic acid microcapsules with ethylcellulose by emulsion solvent evaporation method yielded 90-94% (Dash et al. 2010) of product yield and comparing with the results in this design, the yield of the product with EC was able to exceed this range of values.

5.2.2 Encapsulation efficiency

Encapsulation efficiency (EE) measures the amount of acetylsalicylic acid that was encapsulated comparing to the initial solution. The EE of was 97.3 ± 0.1% for formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%), 89.1 ± 0.6% for formulation 2 (Stock solution; PCL;

DCM; Acidified PVA 1%), and 99.6 ± 0.3% for formulation 3 (Stock solution; PLGA; DCM; Acidified PVA 1%). The highest ratio was obtained in formulation 3 (1 mL of internal aqueous phase; acidified PVA 1%; PLGA; DCM) and the lowest ratio was obtained for formulation 2 (1 mL of internal aqueous phase; acidified PVA 1%; PCL; DCM). The data obtained suggest that the polymer used could be considered the predominant factor that influences the EE of the process since the type of polymer is directly associated to the amount of ASA available to microencapsulate. Therefore, in formulation 3, the polymer PLGA was used while in formulation 2 and 1, the polymer PCL and EC, respectively, was used, suggesting that EE values increase with the hydrophilicity of the polymer enhanced the stability of the primary emulsion and it contributed to such an increase, allowing to conclude that more ASA is available to encapsulate is associated to higher encapsulation efficiency. The use of hydrophilic polymers (PLGA) which carried free carboxylic end groups results in a significantly higher encapsulation efficiency compared to that of an end-capped polymer. The agitation during microencapsulation process can also affect encapsulation efficiency, however it was not relevant for this study (Jyothi et al. 2010).

One study encapsulated acetylsalicylic acid capsules with chitosan by ionic gelation technology and achieved 37-90 % encapsulation efficiencies (Shi et al. 2014). Another study, in which they prepared acetylsalicylic acid -loaded polycarbonate microspheres by a solvent evaporation technique, found EE values in the range of 45-87 % (Thanoo et al. 1993b). Compared with the values obtained in this study, these results are lower, suggesting that the double emulsion solvent evaporation technique is more efficient and effective in the microencapsulation of acetylsalicylic acid.

5.2.3 Loading

Loading measures the amount of acetylsalicylic acid in the microparticle. In this study were found values of 3.7 ± 0.4%, 5.5 ± 1.2%, and 5.0 ± 0.3% for the loading of the final acetylsalicylic acid microparticles in formulations 1 (Stock solution; EC; DCM; Acidified PVA 1%), 2 (Stock solution; PCL; DCM; Acidified PVA 1%), and 3 (Stock solution; PLGA; DCM; Acidified PVA 1%), respectively. Whereas in the last three formulations this parameter is in a range between 5.0 ± 0.3% and 5.5 ± 1.2%, in formulation 1, the loading result was lower but not dissimilar. One explanation may be that it always uses the same solvent, and since DCM is highly immiscible in water, more water molecules may be retained in the bulk of microparticles. By deduction of equations 3 and 5 it was possible to verify that the higher the PY the lower the loading, and this result is verifiable.

5.2.4 Particle Size Distribution

Particle size and size distribution are important parameters for the evaluation of the microparticles and for the development of microencapsulation in the pharmaceutical area

because they influence the encapsulation of the active compound (loading), the drug release profile and the stability of the compound active within them. Particle size and size distribution may also influence the *in vivo* distribution, biological fate, toxicity, and targeting capabilities of the microparticle systems. The result of the particle size distribution is shown in Figure 13 and Table 12, described with more detail in the Appendix C. A lower polydispersity degree and smaller size of microparticles are normally preferred.

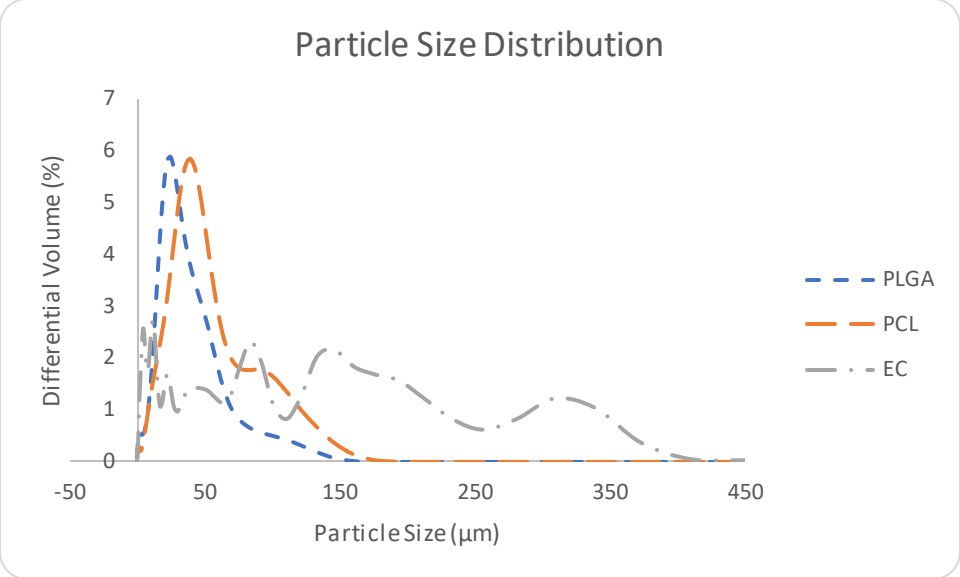


Figure 13 - Size Distribution of Acetylsalicylic Acid Microparticles in different polymers

All the studies already evidenced in this study on the microencapsulation of acetylsalicylic acid obtained results of particle size superior to those of this study, which shows that the technique and all the polymers used were efficient in microencapsulation.

Table 12 - Particle mean diameter and polydispersitivity degree results for the three formulations

	Formulation 1		Formulation 2		Formulation 3	
Parameters	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± s)**	**RSD (%)**	**Mean [a] (M ± s)**	**RSD (%)**
Mean (µm)	53.4 ± 17.8	33.3	37.8 ± 2.5	6.5	27.6 ± 3.1	11.1
SPAN	11.4 ± 0.5	4.0	2.3 ± 0.1	5.0	2.2 ± 0.1	5.0

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

These parameters were evaluated by laser granulometry. The mean diameter values obtained in this study were: 53.4 ± 17.8 µm for formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%); 37.8 ± 2.5 µm for formulation 2 (Stock solution; PCL; DCM; Acidified PVA 1%), and 27.6 ± 3.1 µm for formulation 3 (Stock solution; PLGA; DCM; Acidified PVA 1%). The

formulation 1, in which the EC polymer was used, was the one that obtained the largest mean particle size. This may be the result of aggregation of the microparticles. According to (Jelvehgari & Hassan Montazam 2012), the fact that DCM is immiscible in water, 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. The increase in the mean particle size can be attributed to the higher amount of ASA present in the microparticles. These results are in agreement with results described by other authors using the $w_1/o/w_2$ double emulsion solvent evaporation method (Sharma et al. 2016).

Regarding the volumes distribution (Figure 13) it is possible to conclude that all the formulations are similar and normally distributed, except in formulation 1 where some disturbances where found out in the curve. Indeed, significantly amounts of larger particles were obtained (confirmed during the practical analysis), which may be explained by aggregation effects. 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 11.4 ± 0.5 in formulation 1 compared to the other formulations were the values were, in general, all similar between each other (2.3 ± 0.1 for formulation 2; 2.2 ± 0.1 for formulation 3). Therefore, it is possible to conclude that in formulation 1 the particles presented different sizes among themselves (bigger polydispersitivity), while in the others formulations (formulation 2 and 3) the particles presented a smaller polydispersitivity degree, showing more similar particle sizes between each other. 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 poly-dispersed particles (Gomes et al. 2011). In the present study, all the dispersity degrees results were very similar, with the exception the formulation 1, leading to the conclusion that the formulations 2 and 3 obtained were monodisperse systems, while that the formulation 1 obtained was polydisperse system.

Several processes and formulation and process parameters are described to affect the particle size distribution as the stirring speed, the loading, polymer concentration, polymer molecular weight, surfactant concentration in the external aqueous phase among others (Krishnamachari et al. 2007). Figure 14 shows the final microparticles after lyophilization.

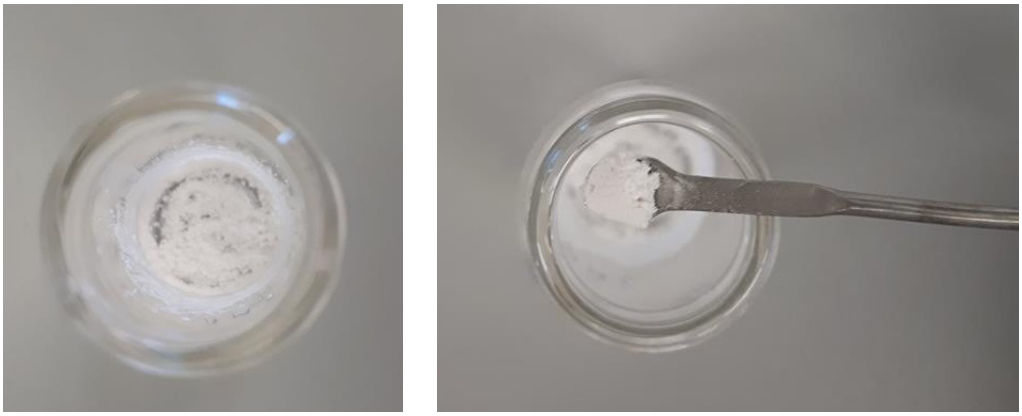


Figure 14 - Dried microparticles obtained

5.2.5 Particles morphology

The ASA microparticles obtained in the present study were analysed by SEM for the respective particle morphology analysis. Figures 15 and 16 show the morphology of EC and PLGA microparticles, respectively, by way of example. EC-ASA microparticles prepared by double emulsion solvent evaporation had a smooth surface and a regular spherical shape. In addition, it is possible to observe microparticles with few pores, monodisperse, and some agglomerates. PLGA-ASA microparticles had a rather rough surface, spherical shape, few pores and polydisperse. Furthermore, it is possible to observe that in Figure 15, a small agglomeration of the microparticles happened. 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). Another interesting conclusion, which is possible to take from a different study, is that it is expected that a smaller concentration of the core ingredient (higher volume) leads to smooth and few porous surfaces (Das & Rao 2007), which was observed in the present study since in formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%), where 5 mL of internal aqueous phase was used, the most smooth, regular and spherical particles where obtain (Figure 15). It should be noted that the MPs of PLGA when taken to the SEM, and the magnification increased, these almost collapsed, whereas with the MPs of EC, the magnification could be increased or decreased, and the shape of these remained. This result demonstrates that the EC polymer has a higher mechanical strength.

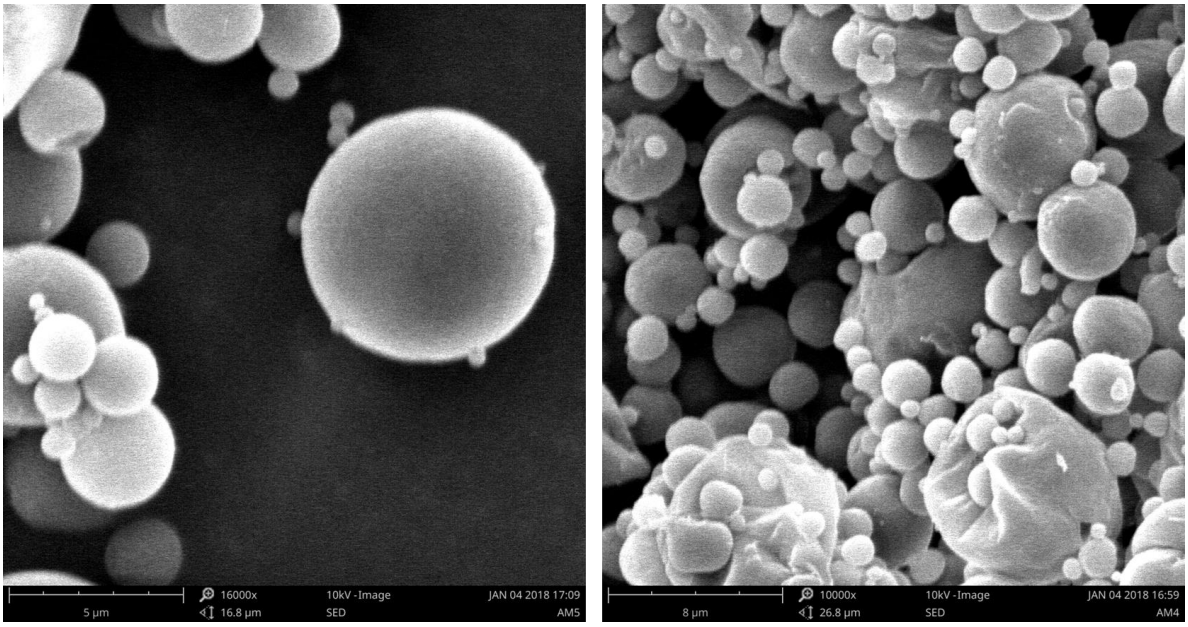


Figure 15 - SEM image of EC-ASA microparticles prepared by $w_1/o/w_2$ solvent evaporation

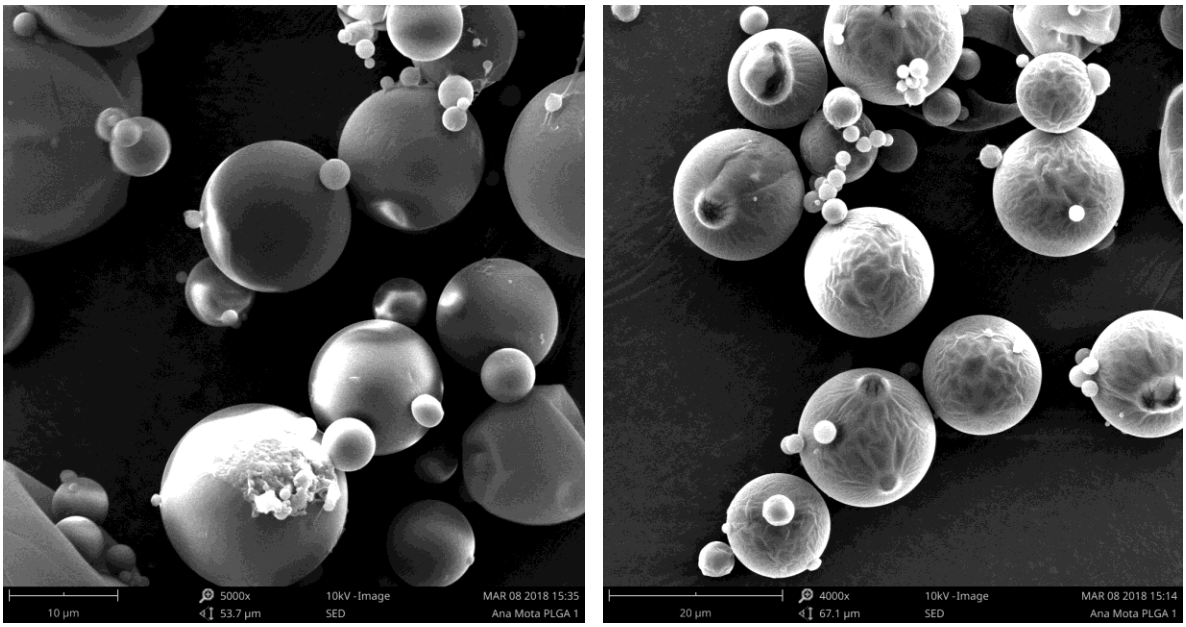


Figure 16 - SEM image of PLGA-ASA microparticles prepared by $w_1/o/w_2$ solvent evaporation

5.3 Controlled release studies

Controlled release is one of the advantages of microencapsulation since it allows the release under the desired conditions, improving compound effectiveness without the need for high dosages (Gupta & Dey 2013). The controlled release study of acetylsalicylic acid from the microparticles obtained was planned to simulate conditions of the gastrointestinal tract and to study the consequent behaviour of the microparticles with the active compound over a period of time, 2 minutes for the salivary fluid and 2 hours for the remaining fluids. The release profile

study was performed on simulated, salivary, gastric and intestinal fluids at pHs 7, 3 and 7, respectively.

The mechanisms by which an active compound can be released is through its diffusion through the pores filled with the release medium with either the dissolution of the polymer being present or diffusion through the polymer matrix wherein in this case the physicochemical properties of the compound have to be compatible with the polymer. Another option is osmotic pressure, which is only possible in non-swelling systems and finally thanks to erosion or degradation of the polymer, not involving the transport of active compound (Gupta & Dey 2013) (Singh et al. 2010).

Instrumental analysis for controlled release studies was performed with the UV-Vis detection spectrophotometer. Moreover, Figures 17, 18 and 19 compare all ASA release profiles from the final microparticles in the three different mediums: SSF, SGF and SIF, respectively.

Therefore, is possible to observe that the mediums were there was more and less release of acetylsalicylic acid were not concordant among the three formulations. It can be seen from Figure 17 that there was no release of the pharmacologically active compound in the SSF medium in formulation 2 (Stock solution; PCL; DCM; Acidified PVA 1%) and 3 (Stock solution; PLGA; DCM; Acidified PVA 1%), with formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%) releasing 0.3% after 2 minutes. This result was expected as the desired is that there is no release of the compound in the mouth and thus it reaches its site of absorption, duodenum. Furthermore, this result can be explained by the fact that in this medium, SSF, there is the α-amylase enzyme which acts on carbohydrates, and since only the EC polymer is a carbohydrate, then there is only release of the active compound in the formulation 1, in which this polymer is present.

When analysing the SGF medium release profiles, in Figure 18 it is possible to conclude that the highest percentage of release after 2 hours was in formulation 3, where the polymer used was PLGA, followed by formulation 2 (PCL) and finally the formulation 1 (EC). When analysing the SIF medium release profile, in Figure 19, acetylsalicylic acid microparticles showed a faster release profile in this medium compared to the release in SGF medium. For this medium, SIF, it is possible to conclude that the highest percentage of release after 2 hours was in formulation 3, where the polymer used was PLGA, followed by formulation 2 (PCL) and finally the formulation 1 (EC). These results were as expected, since the pharmacologically active compound, ASA, is absorbed in the upper part of the small intestine, duodenum, and therefore further release thereof, into the simulated intestinal fluid was as intended.

The highest percentages of release of the compound in both SGF and SIF medium were obtained in formulation 3, in which the microparticles were coated with PLGA (Figure 18 and 19). This may be due to the degradation rate of the polymer. PLGA is more hydrophilic

compared to PCL and EC, which lacks glycolide acid, being more hydrophobic in nature, and therefore has a lower degradation rate ((Thi & Lee 2010),(Sharma et al. 2016)). The release of acetylsalicylic acid in SGF was slow which could be due to poor solubility of the drug and polymer in acidic medium. In SIF, there was abrupt release of the drug indicating that the medium can diffuse into polymeric matrix and dissolve the drug (Dash et al. 2010).

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 greater absorption of the compound. Therefore, those suggestions were confirmed in our study. Indeed the formulation 3 was the formulation with smaller particles (27.6 μm) and, consecutively, higher release profiles: 1.8% for SGF and 9.4% for SIF.

In the present study, although some unusual results were observed, it can generally be summarized that the ASA release profiles obtained for SGF and SIF, except for formulation 1 presented a first initial burst release, possibly due to a rapid release of ASA molecules which are adsorbed outside the microparticle, or to a rapid release of microencapsulated ASA which is closed to the polymeric matrix surface being easily release.

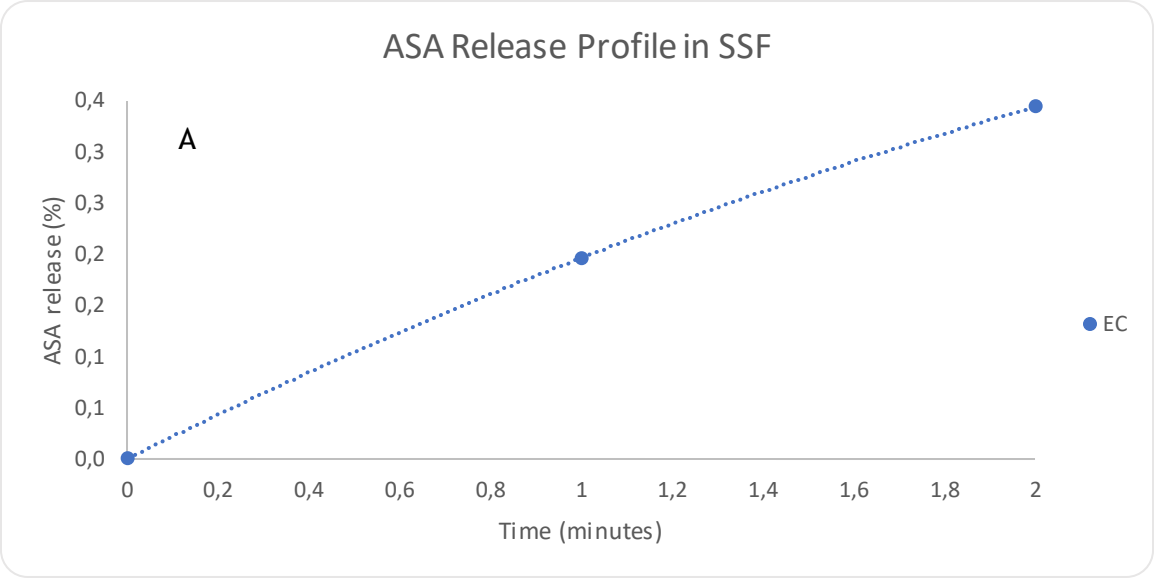


Figure 17 - Results of the controlled release study, in SSF (pH 7.0), for the formulation 1

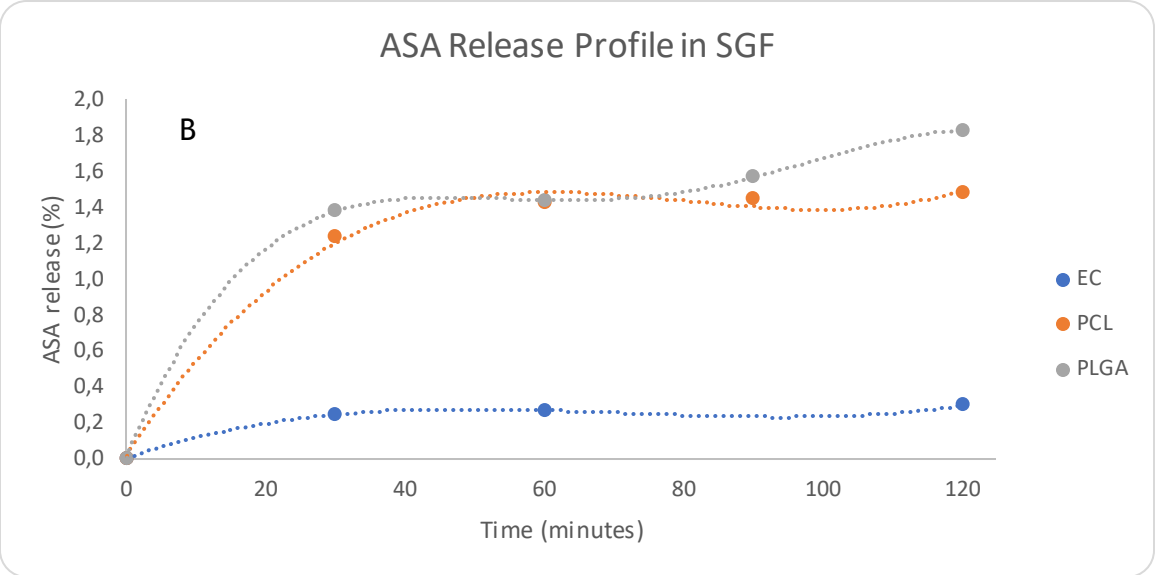


Figure 18 - Results of the controlled release study, in SGF (pH 3.0), for the three microparticles formulations

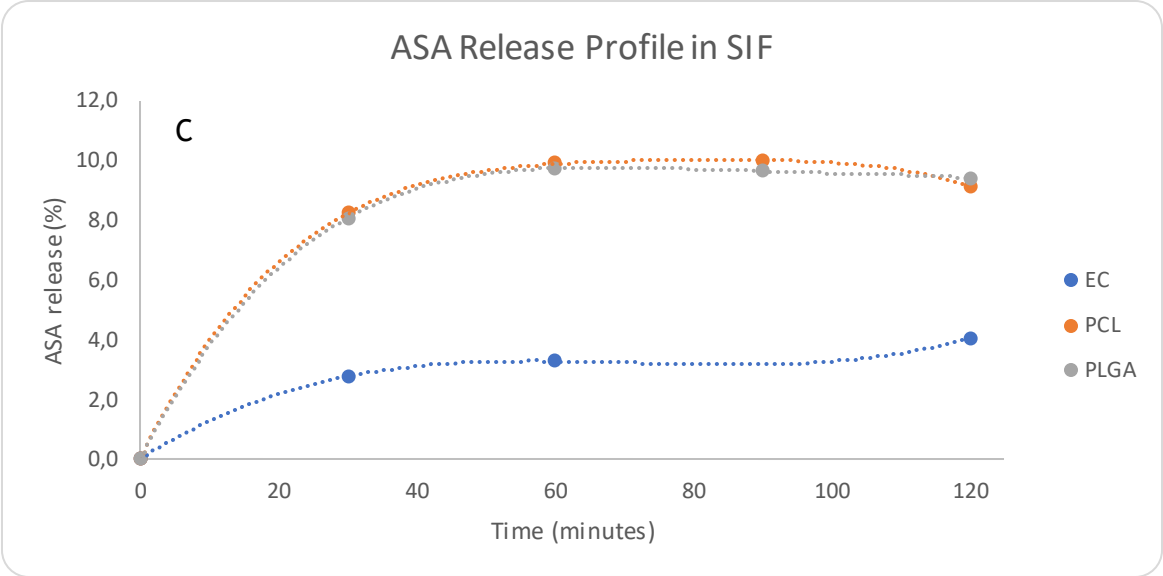


Figure 19 - Results of the controlled release study, in SIF (pH 7.0), for the three microparticles formulations

They show that the fastest release profile is in the intestinal fluid, as intended, as the compound is to be absorbed in its active form in the duodenum. In addition, although the pH in the salivary and intestinal fluid is the same and the release profiles of the compound are different, it does not determine that the pH is indeterminate for the controlled release studies. Studies show that the pH and composition of the respective release media are determinant in

the drug release profiles and in this study the salt concentrations in the salivary and intestinal media are different, which determines different release profiles of the pharmacologically active compound (Faisant et al. 2006).

6 Conclusions

In this project, the acetylsalicylic acid was microencapsulated with ethylcellulose, polycaprolactone and poly(lactide-co-glycolide acid) by the double emulsion solvent evaporation method, in order to overcome the limitations of the therapeutic agent. In this study, in addition to the $w_1/o/w_2$ technique, the s/o/w technique was also used, however, the results obtained for the latter technique were not consistent with the literature. The microparticles were characterized by encapsulation efficiency, product yield, loading, particle size distribution, surface morphology, as well as the controlled release profile. Regarding the results about these parameters, the maximum product yield was 98.3 $\pm$ 3.0% in formulation 1 (Stock solution; EC; DCM; Acidified PVA 1%); the maximum encapsulation efficiency was 99.6 $\pm$ 0.3% in formulation 3 (Stock solution; PLGA; DCM; Acidified PVA 1%); and the loading was found out to be maximum in formulation 2 (Stock solution; PCL; DCM; Acidified PVA 1%), 5.5 ± 1.2%. Additionally, the prepared microparticles showed sizes ranging between 27.6 ± 3.1 µm and 53.4 ± 17.8 µm for the overall formulations tested, and the lower SPAN value obtained was 2.2 $\pm$ 0.1 for formulation 3. The microparticles were generically spherical, monodisperse, few porous and superficially smooth.

Moreover, a UV-Vis Spectrophotometry method for acetylsalicylic acid determination and quantification was developed and validated. All parameters were within the conditions proposed, indicating that this method was sensitive, linear, and precise, with low detection and quantification limits, in the three mediums investigated. The proposed method was used to predict the encapsulation efficiency and the release profile of acetylsalicylic acid from the final particles.

In addition, this study investigated the controlled release profile of ASA in simulated fluids, namely salivary, gastric and intestinal fluids. The release was higher in intestinal fluid simulated comparing to gastric fluid simulated. For instance, formulation 3 was the one showing highest release after 2 hours in SGF (1.8%) and SIF (9.8%), showing that the PLGA polymer is the polyester most easily hydrolyzed in acid and basic medium. We might assume that the formulation parameters used in formulation 3 could be our optimized conditions because those particles obtained the higher release profile in intestinal fluid (area of absorption of the ASA) allowing its application in the pharmaceuticals formulations.

Microencapsulation of acetylsalicylic acid into microparticles was possible, allowing controlled release of the drug. This project proves the success of the microencapsulation of acetylsalicylic acid for therapeutic application, however, further studies should be carried out.

7 Future Work and Limitations

Several drawbacks emerged during the development of this work, which limited the obtained results. Nevertheless, proposed laboratorial work for this project was successfully completed. The availability of the equipment (coulter counter and freeze-dryer were shared with others researchers and students), and the fact that the UV-Vis spectrophotometer does not have the best conditions, were the major limitations that delayed or influence the obtainment of results in the present project. Initially, the stock solution of acetylsalicylic acid was placed in the refrigerator, however it formed a precipitate in powder form and was subsequently placed at room temperature to avoid precipitation.

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 acetylsalicylic acid more effectively, because for pharmaceuticals applications a slower release is intended. Moreover, it is suggested the determination of the minimum amount ingested to achieve the desired effect (analgesic). The future work is to adapt this proven success to decrease the environmental problems of massive and irrational use of analgesics. As preliminary tests, the results of this present study suggested the success of acetylsalicylic acid microencapsulation.

Microencapsulation can be a promising solution to decrease the increasing problem of high analgesics dosages. The development of safe and efficient microencapsulation techniques will require, in the future, in-depth investigations of both the biological and technological aspects of these systems.

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Appendix

A. Scheme for the synthesis of acetylsalicylic acid

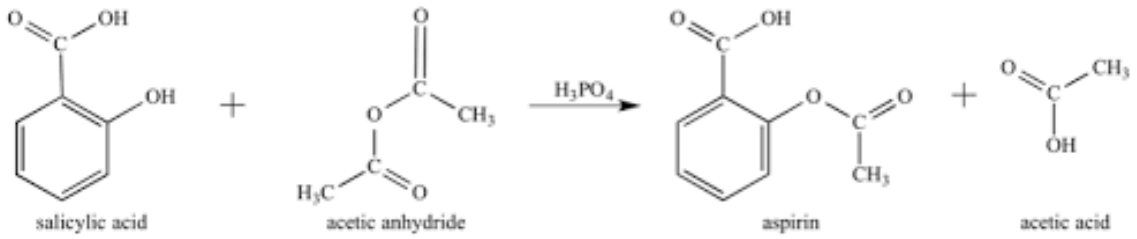
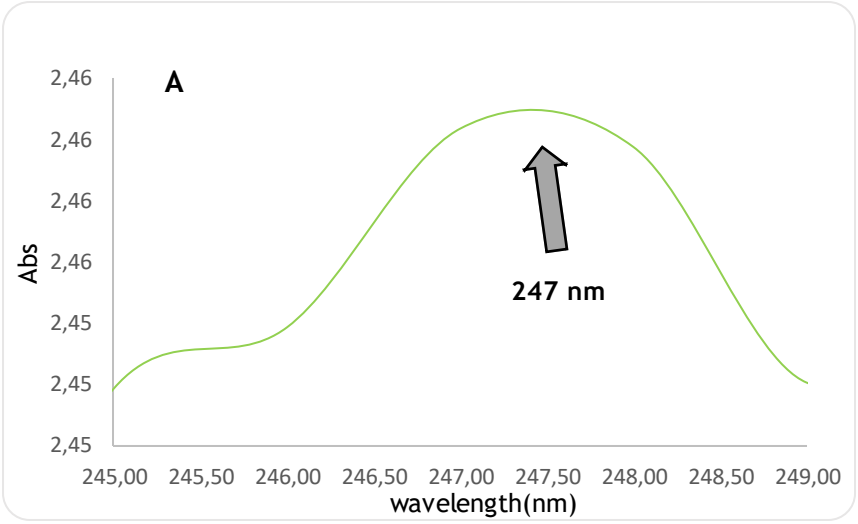
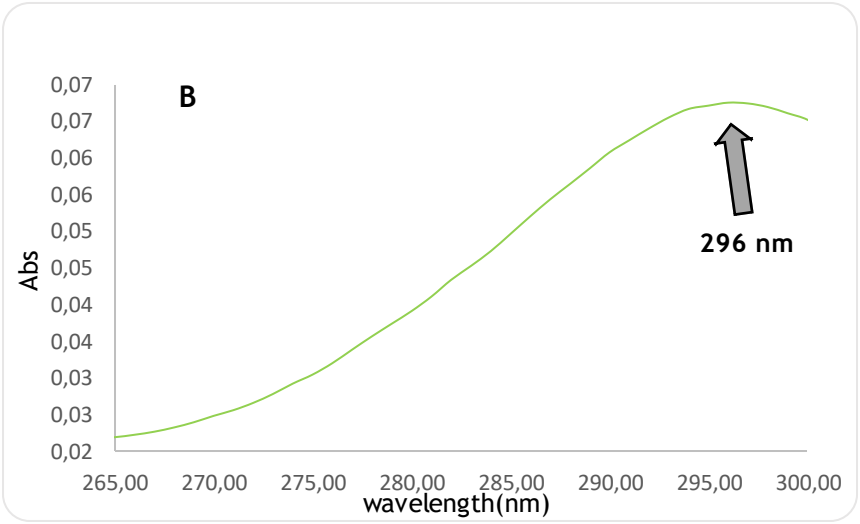


Figure A1 - Scheme for the synthesis of acetylsalicylic acid

B. Spectrums of absorption of acetylsalicylic acid for differents fluids





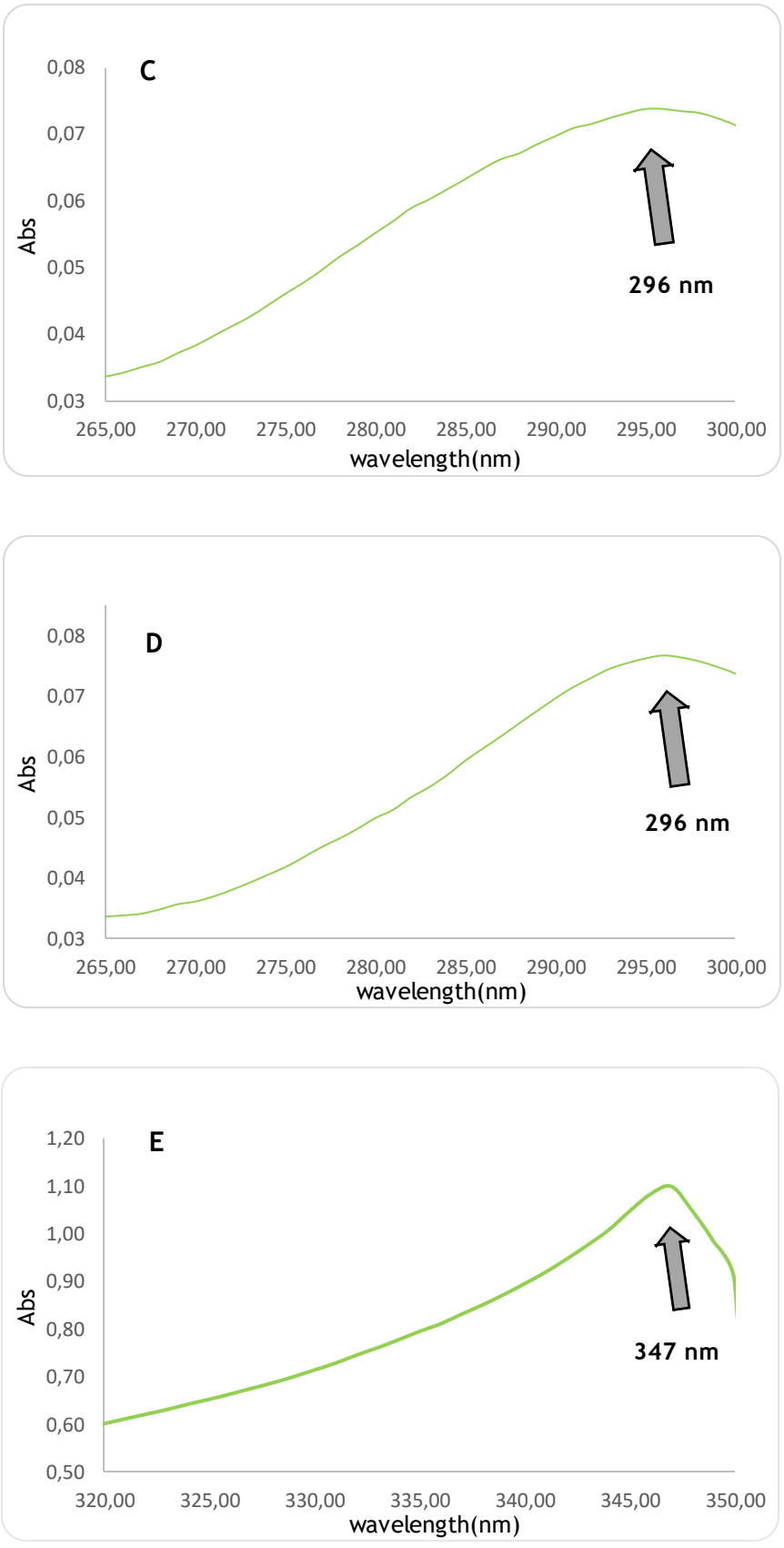


Figure B1 - Spectrum of absorption of acetylsalicylic acid for ultrapure water, different simulated fluids and acidified acid, a) ultrapure water at pH 2, b) SSF at pH 7, c) SGF at pH 3, d) SIF at pH 7 and e) acidified PVA

C. Particle Size Distribution

Table C1 - Particle Size Distribution Analysis for Formulation 1

Parameters	EC1	EC2	EC3
D10	1.9	2.4	2.4
D50	8.9	16.6	18.5
D90	108.2	189.5	206.5
Mean (μm)	33.0	61.3	65.8
SPAN	11.9	11.3	11.0

Table C2 - Particle Size Distribution Analysis for Formulation 2

Parameters	PCL1	PCL2	PCL3
D10	1.2	5.0	4.8
D50	31.6	33.8	33.4
D90	69.2	86.4	84.6
Mean (μm)	34.3	39.9	39.2
SPAN	2.2	2.4	2.4

Table C3 - Particle Size Distribution Analysis for Formulation 3

Parameters	PLGA1	PLGA2	PLGA3
D10	2.3	3.8	3.7
D50	21.5	24.7	24.5
D90	46.2	60.5	59.2
Mean (μm)	23.3	30.1	29.5
SPAN	2.0	2.3	2.3

D. Equipment used in this project

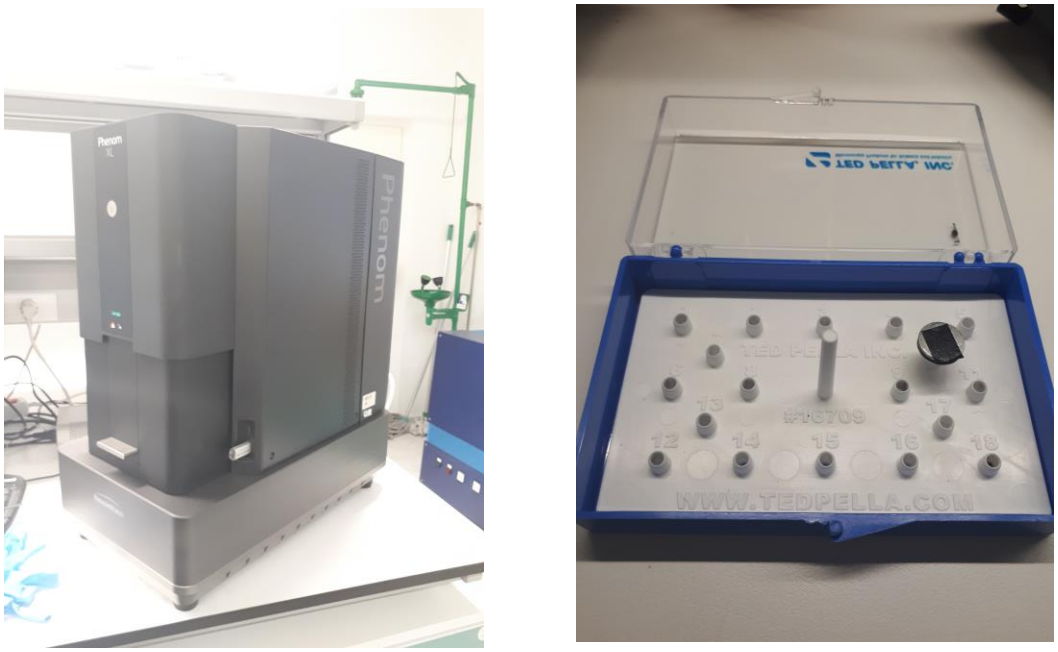
In this appendix are presented some of the most important equipment used for the right execution of this project, including the emulsification equipment (the high-performance homogenizer) (Figure D1) for microspheres formulation. It is also presented the equipment used to freeze-dry microspheres (Figure D2). Moreover the equipment required for microspheres quality control analysis namely the coulter counter and the scanning electron microscope are

presented (Figure D3 and D4). Additionally, is presented the equipment used for the validation of analytical methods and to obtain the encapsulation efficiency (Spectrophotometer UV-Vis V-530) (Figure D5) and still the equipment used to obtain the microparticles (vacuum filtration system) (Figure D6). The equipment used for pH measurements is also presented (Figure D7).

Figure D1 - High-performance homogenizer

Figure D2 - Freeze-dryer

Figure D3 - Coulter Counter-LS 230 Particle Size



(A) ***(B)***

Figure D4 - Scanning electron microscope

((**A**) – The equipment of scanning electron; **(B)** - The samples were sputter-coated with gold)

Figure D5 - Spectrophotometer UV-Vis V-530

Figure D6 - Vacuum filtration system

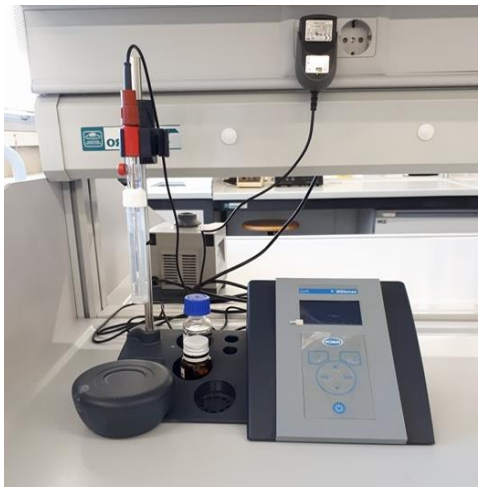


Figure D7 - pH meter