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Preliminary studies of coffee natural antioxidants microencapsulation and food application

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“Nothing in life is to be feared; it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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ABSTRACT

Consumption alone of foods naturally rich in antioxidants is not enough to reduce oxidative stress associated with cancer, diabetes and cardiovascular diseases. Therefore, functional foods fortified with antioxidants are gaining more popularity. However, natural antioxidants are sensitive to heat, light and oxygen, which limit their application in the food industry. Microencapsulation might overcome these problems since it is able to protect the antioxidant from degradation, mask its taste and control its release. Coffee is considered the most consumed and commercialized food product in the world and also a rich source of antioxidants such as chlorogenic acid (CGA) and caffeic acid (CAF). The aim of this work was to microencapsulate the aforementioned coffee antioxidants by spray-drying for future food applications using sodium salt carboxymethyl cellulose (Na-CMC) as a coating agent. Resulting microparticles were characterized by its process product yield, particle size distribution (by laser granulometry), morphology (by scanning electron microscopy), controlled release (in water at pH 5.6 and pH 2) and antioxidant activity (by ABTS assay). Analytical methods (UV-Vis spectrophotometry) were developed for the quantification of CGA and CAF during release. CGA calibration curve was linear from 0.50 to 14 $mg.L^{-1}$ and CAF calibration curve was linear from 0.25 to 15 $mg.L^{-1}$. The limit of detection was 0.10 $mg.L^{-1}$ for CGA and 0.29 $mg.L^{-1}$ for CAF while the limit of quantification was 0.34 $mg.L^{-1}$ for CGA and 0.96 $mg.L^{-1}$ for CAF. Both methods were considered precise (repeatability and intermediate precision values below 5%, except for the lowest concentration) and accurate (accuracy values close to 100%). Product yield was 37.6% and 40.8% for microparticles loaded with CGA and CAF, respectively. Particle size distribution found that microparticles loaded with CGA presented mean diameters of 8.37 μm (considering volume distribution) and 0.44 μm (considering number distribution). For microparticles loaded with CAF mean diameters were 8.78 μm (considering volume distribution) and 0.41 μm (considering number distribution). Particle morphology assessment revealed small spherical shape microparticles with smooth surfaces for all samples. Controlled release profiles were similar for all samples but were influenced by microparticles core composition (faster release for CAF than CGA) and also by medium pH (faster release at pH 2 than pH 5.6). Total release times ranged from 30 min to 2 h, which are satisfactory considering the average digestion time. Antioxidant activity was higher after the spray-drying process, showing that the chosen methodology does not compromise CGA and CAF antioxidant capacity. As preliminary studies, the results presented in this work show the potential of spray-dried Na-CMC microparticles as carriers of coffee antioxidants. However, further studies may be explored for better suitability of said microparticles for food applications.

Keywords: antioxidants, caffeic acid, carboxymethyl cellulose, chlorogenic acid, coffee, controlled release, food applications, microencapsulation, spray-drying.

RESUMO

A popularidade de alimentos fortificados com antioxidantes tem vindo a crescer uma vez que o consumo de alimentos naturalmente ricos em antioxidantes não é suficiente para reduzir o stress oxidativo associado ao cancro, diabetes e doenças cardiovasculares. No entanto, os antioxidantes naturais são sensíveis à luz, calor e oxigénio, limitando a sua aplicação na indústria alimentar. A microencapsulação pode solucionar estes problemas uma vez que é capaz de proteger o antioxidante da degradação, disfarçar o seu sabor e controlar a sua libertação. O café é um dos produtos alimentares mais consumidos e comercializados e também uma boa fonte de antioxidantes naturais como o ácido clorogénico (CGA) e ácido cafeico (CAF). O objetivo deste trabalho consistiu na microencapsulação através de secagem por atomização dos antioxidantes mencionados utilizando a carboximetil celulose sódica (Na-CMC) como agente encapsulante. As micropartículas resultantes foram caracterizadas relativamente ao rendimento do processo, à distribuição do tamanho (por granulometria de laser), à morfologia (por microscopia eletrónica de varrimento), à libertação controlada (em água a pH 5.6 e pH 2) e à atividade antioxidante (por ensaio ABTS). Métodos analíticos de espetrofotometria UV-Vis foram desenvolvidos para a quantificação de CGA e CAF durante o estudo de libertação controlada. A curva de calibração do CGA foi linear entre 0,50 e 14 $mg.L^{-1}$ e a do CAF foi linear entre 0,25 e 15 $mg.L^{-1}$. O limite de deteção foi 0,10 $mg.L^{-1}$ para o CGA e 0,29 $mg.L^{-1}$ para o CAF enquanto o limite de quantificação foi 0,34 $mg.L^{-1}$ para o CGA e 0,96 $mg.L^{-1}$ para o CAF. Ambos os métodos foram considerados precisos (com valores de repetibilidade e precisão intermédia inferiores a 5%, exceto para a concentração mais baixa) e exatos (com valores de exatidão próximos de 100%). O rendimento do processo foi 37,6% e 40,8% para micropartículas com CGA e CAF, respetivamente. Partículas com CGA apresentaram tamanhos médios de 8,37 μm (considerando distribuição em volume) e 0,44 μm (considerando distribuição em número). Partículas com CAF apresentaram tamanhos médios de 8,78 μm (considerando distribuição em volume) e 0,41 μm (considerando distribuição em número). Todas as micropartículas apresentaram morfologia pequena, com forma esférica e superfície lisa. Os perfis de libertação controlada foram semelhantes para todas as formulações, mostrando dependência tanto da composição do núcleo (libertação mais rápida para CAF do que para CGA) como do pH do meio (libertação mais rápida a pH 2 do que a pH 5,6). Os tempos de libertação total variaram entre 30 min a 2 h, sendo considerados satisfatórios comparando com o tempo médio de digestão. A atividade antioxidante foi maior após o processo de microencapsulação do que em soluções livres, mostrando que a metodologia escolhida não compromete a capacidade antioxidante do CGA e CAF. Como estudos preliminares, os resultados apresentados neste trabalho mostraram o potencial de micropartículas de Na-CMC obtidas através de secagem por atomização como fornecedores de antioxidantes do café. No entanto, será necessário a realização de mais estudos de modo a melhorar a funcionalidade destas micropartículas para aplicações alimentares.

Palavras-chave: antioxidantes, aplicações alimentares, ácido cafeico, ácido clorogénico, café, carboximetil celulose, libertação controlada, microencapsulação, secagem por atomização.

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GLOSSARY

%R	Percent recovery
a	Sensitivity
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AU	Absorbance units
b	Intercept of the regression
CAF	Caffeic acid
CAS	Chemical abstracts service
CD (or β-CD)	Cyclodextrin (or beta-cyclodextrin)
CFQA	Caffeoyl feruloylquinic acid
CGA	Chlorogenic acid
CMC	Carboxymethyl cellulose
CQA	Caffeoylquinic acid
CV	Coefficient of variation
DE	Dextrose equivalent
diCQA	Dicaffeoylquinic acid
diFQA	Diferuloylquinic acid
di-*p*-CoQA	Di-*p*-coumaroylquinic acid
DNA	Deoxyribonucleic acid
DR	Degree of reaction
DS	Degree of substitution
E	Europe
EE	Entrapment efficiency
FQA	Feruloylquinic acid
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
HP-β-CD	Hydroxypropyl-beta-cyclodextrin
LOD	Limit of detection
LOQ	Limit of quantification
Na-CMC	Sodium salt carboxymethyl cellulose
p-CoQA	*p*-coumaroylquinic acid
PY	Product yield
R	Linearity coefficient
s	Standard deviation
SEM	Scanning electron microscopy
SLN	Solid lipid nanoparticles
T	Temperature
TE	Trolox equivalent
UV-Vis	Ultraviolet visible

CHAPTER 1: BACKGROUND MOTIVATION AND PROJECT GUIDELINE

1.1. Background motivation

Consumers nowadays are concerned about their health and leading a healthy life, showing interest in foods that contain bioactive or functional ingredients (especially natural ones) that increase food nutritional value and health status (Fu et al., 2011; Hygreeva, Pandey, & Radhakrishna, 2014). Recent studies confirm that one of the main causes behind premature aging and dangerous diseases like cancer, cardiovascular diseases, neurodegenerative diseases (Alzheimer and Parkinson) (Stelmach, Pohl, & Szymczycha-madeja, 2015) or diabetes lies on the excessive accumulation in the human body of products derived from oxygen and nitrogen reactions such as superoxide anion, hydroxyl radical and hydroperoxyl radical (Yashin, Yashin, Wang, & Nemzer, 2013). The constant buildup of free radicals causes oxidative stress conditions, damaging DNA molecules, blood vessels walls, proteins and lipids. More specifically, the cleavage of DNA molecules influences cell growth regulation, resulting in the formation of cancerous cells. Also, cardiovascular diseases may be a result of the accumulation of oxidized low-density lipoproteins on blood vessels walls (Yashin et al., 2013).

Antioxidant therapy is a powerful tool to reduce oxidative stress in the body since these compounds are able to terminate or delay the chain reaction caused by free radicals (Oroian & Escriche, 2015). By consuming natural antioxidants present in foods such as vegetables, fruits, berries, grains, among others (Sies, 1993; Yashin et al., 2013), the appearance of various common diseases may be prevented. However, consumers do not always prefer the aforementioned foods and their consumption alone might not be enough to obtain the necessary intake of antioxidants. Thus, functional foods fortified with antioxidants could overcome the presented problem, providing a higher amount of antioxidants.

The major categories of antioxidants found in food include vitamins (e. g. vitamin C and vitamin E), carotenoids (e. g. carotenes and xanthophylls) and polyphenols (e. g. flavonoids, phenolic acids, lignans and stilbenes). However, they may present different mechanisms of action, namely: scavenge of peroxidation-inducer species, chelation of metal ions in order to stop them from creating reactive species and from degrading peroxide, O_2• mitigation in order to prevent peroxides formation, interruption of the auto-oxidative chain reaction and attenuation of areas of high O_2 concentrations. Besides their antioxidant capacity, antioxidants are often associated with other beneficial effects such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic

properties and gut microbial health (Bartoszek & Polak, 2012; Boffetta et al., 2010; Cardona, Andres-Lacueva, Tulipani, Tinahones, & Queipo-Ortuno, 2013; Diaz et al., 2012; Oroian & Escriche, 2015).

As mentioned before, there are food products which are naturally rich in antioxidants. Coffee is considered the most consumed and commercialized food product in the world and the second most traded commodity, after petroleum (Murthy & Naidu, 2012). Regardless of its controversial effects on human health, coffee is acknowledged as one of the key sources of antioxidants (such as chlorogenic and caffeic acids) and other bioactive compounds in the daily diet of population worldwide (Cano-Marquina, Tarín, & Cano, 2013; Farah, 2012).

Despite their beneficial effects, natural antioxidants are usually easily oxidized and sensitive to heat and light, which limit their application in the food industry (Chao, Wang, Zhao, Zhang, & Zhang, 2011; Lozano-Vazquez et al., 2015). Also, some of these compounds show other limitations such as unpleasant taste, poor availability and also high susceptibility to storage and processing conditions and gastrointestinal environments (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011; Poshadri & Kuna, 2010; Wilson & Shah, 2007).

In many cases, microencapsulation is a reliable technique that might overcome these challenges since it is able to preserve stability of the bioactive compounds during processing and storage, improve the retention time of the nutrient in the food, allow controlled and targeted release, prevent undesirable interactions with food matrix, slow down the degradation processes (e.g., oxidation or hydrolysis), mask bad tasting or smell and increase bioavailability, while maintaining the bioactive component functionality. Other advantages might include ease of handling, adequate concentration and uniform dispersion (Lozano-Vazquez et al., 2015; Nedovic et al., 2011; Wilson & Shah, 2007).

Therefore, the development of food applications containing microencapsulated coffee antioxidants shows great potential since it is capable of providing the natural benefits offered by antioxidants while protecting them from degradation.

1.2. Thesis objectives

The main objectives of the current project were the microencapsulation of coffee antioxidants, namely chlorogenic and caffeic acids, characterization of resulting microparticles, their controlled release and antioxidant activity assessment. Spray-drying was used as the microencapsulation method due to its flexibility, low cost and availability. The use of dietary fibers as encapsulating agent (namely carboxymethylcellulose) was also an important goal of this work since they promote beneficial physiological effects.

Specific aims included evaluation of the spray-drying process by its product yield, microparticles characterization by particle size distribution, morphology and controlled release. Since the desired target-location for the microparticles release was the gastrointestinal tract, controlled release assays were performed in water at pH 5.6 and also pH 2 in order to approximate the acidic conditions experienced in the stomach. Validation of analytical method (UV-Vis spectrophotometry) was performed in order to quantify the controlled release of chlorogenic and caffeic acids. Entrapment efficiency was also determined through the controlled release assays. Moreover, antioxidant activity was measured to verify whether the microencapsulation process resulted in the loss of antioxidant capacity.

1.3. Organization and structure

Present thesis is divided in 7 chapters. Chapter 1 mentions the project background motivation, its main objectives as well as it organization and structure. Chapter 2 includes a theoretical introduction to the topics and procedures discussed along the thesis, such as coffee antioxidants, microencapsulation concepts, techniques and encapsulating agents (with special emphasis to spray-drying and carboxymethyl cellulose). Chapter 3 comprises an overall review of the state of the art so far regarding the encapsulation of chlorogenic and caffeic acids, providing some comments and simple statistics. Chapter 4 describes the materials and methods required for the implementation of this project. Chapter 5 shows the main results, particularly, the validation of the analytical method used for chlorogenic and caffeic acid quantification (UV-Vis Spectrophotometry); the spray-drying product yield; microparticles characterization by particle size distribution and morphology; controlled release profiles for chlorogenic and caffeic acid in water at pH 5.6 and pH 2; entrapment efficiency and antioxidant activity. Finally, chapter 6 contains the project main conclusions and chapter 7 reveals some limitations and future work suggestions. Bibliography used during the development of this work may be consulted at the References section and additional information may be consulted in the Appendix section.

CHAPTER 2: INTRODUCTION

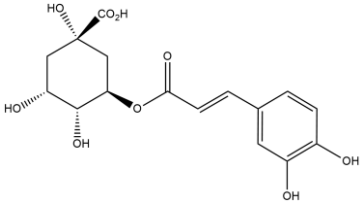
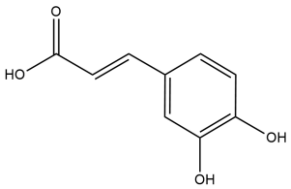
2.1. Chlorogenic and caffeic acids in coffee

Coffee tree (Rubiaceae family, genus *Coffea*) has more than 80 identified species, although only two are of economic interest: *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) (Farah, 2012). Arabica is responsible for approximately 75% of the world production, reaching higher prices since it is considered superior due to its sensory properties (milder and flavorful taste). Robusta is more acid but more resistant to plagues and is associated with the instant coffee industry (Mussatto, Machado, Martins, & Teixeira, 2011; Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013).

Like most plants, green coffee beans major components are insoluble polysaccharides such as cellulose and hemicelluloses, accounting for approximately 50% of the weight. Nevertheless, a variety of soluble carbohydrates (e. g. fructose, glucose, galactose, arabinose and sucrose) are also present in coffee beans and are responsible for aroma binding, foam stabilization and extract viscosity. Other constituents include non-volatile aliphatic acids (such as citric and quinic acids), volatile acids (such as acetic acid), phenolic species (e.g. caffeine, caffeic and chlorogenic acids), oils and waxes, protein and free amino acids, and minerals (Arya & Rao, 2007; Bicchi, Binello, Pellegrino, & Vanni, 1995; Esquivel & Jiménez, 2012; Fischer, Reimann, Trovato, & Redgwell, 2001; Gonzalez-Rios et al., 2007; Naidu, Sulochanamma, Sampathu, & Srinivas, 2008; Stelmach et al., 2015; F. Wei et al., 2012). Furthermore, coffee beans contain a diversity of bioactive compounds that can interact with the human body in a complex way, resulting in many beneficial outcomes, namely improvement of antioxidant and scavenging properties, neural system stimulation (Stelmach et al., 2015) and also weight management through metabolism acceleration (Shimoda, Seki, & Aitani, 2006). It has been acknowledged that beneficial effects from coffee consumption are related to its high content of antioxidants such as chlorogenic, ferulic, caffeic and coumaric acids for green coffee beans and also melanoidins for roasted coffee beans (Yashin et al., 2013), being that chlorogenic and caffeic acids are considered the most relevant markers in coffee samples (Brezová, Šlebodová, & Staško, 2009; Cämmerer & Kroh, 2006). Although these are the main contributors, other compounds present in coffee such as caffeine, trigonelline and phenylalanines (formed during coffee roasting) also show antioxidant properties (Farah & Donangelo, 2006). Parameters like plant species, climate, technological production aspects (e.g. roasting conditions), among others may influence the content of antioxidants in coffee beans (Amare & Admassie, 2012; Kilmartin, 2003; Oliveira-neto et al., 2016; Vignoli, Viegas, Bassoli, & Benassi, 2014).

Table 1 shows some of the properties and chemical structures of chlorogenic and caffeic acids.

Table 1. Chlorogenic acid and caffeic acid characteristics and chemical structure.

Compounds	Chlorogenic acid	Caffeic acid
CAS number	327-97-9	331-39-5
Synonym	3-Caffeoylquinic acid	3,4-Dihydroxycinnamic acid
Molecular Formula	$C_{16}H_{18}O_9$	$C_9H_8O_4$
Chemical Structure		
Molecular Weight (g.mol^{-1})	354.31	180.16
Solubility in water (g.L^{-1})	3.44	1.61
Melting Point (ºC)	205	225

Adapted from HMDB and PubChem, 2016.

The most common polyphenols in coffee are phenolic acids mainly caffeic acid, a type of trans-cinnamic acid, and its derivative, chlorogenic acid. Chlorogenic acids (CGAs) are phenolic compounds formed by the esterification of trans-cinnamic acids with quinic acid (Figure 1A), that may exist in different isomeric forms depending on the position of the ester bond (Figure 1B). These compounds confer astringency, bitterness and acidity to the coffee brew. The main types of chlorogenic acids in coffee are caffeoylquinic acids (CQA, which represent 80% of total chlorogenic content), dicaffeoylquinic acids (diCQA), feruloylquinic acid (FQA), *p*-coumaroylquinic acids (*p*-CoQA) and caffeoyl feruloylquinic acids (CFQA). However, other minor classes such as diferuloylquinic acids (diFQA), di-*p*-coumaroylquinic acids (di-*p*-CoQA), dimethoxycinnamoylquinic acids and others are also found in less than 1% of total chlorogenic content. While 3-O-caffeoylquinic acid (3-CQA) is customarily recognized as chlorogenic acid, isomers of CGA include 4-O-caffeoylquinic acid (cryptochlorogenic acid or 4-CQA) and 5-O-caffeoylquinic acid (neochlorogenic acid or 5-CQA) (Figure 1B) (Cano-Marquina et al., 2013; Farah & Donangelo, 2006; Perrone, Farah, Donangelo, de Paulis, & Martin, 2008).

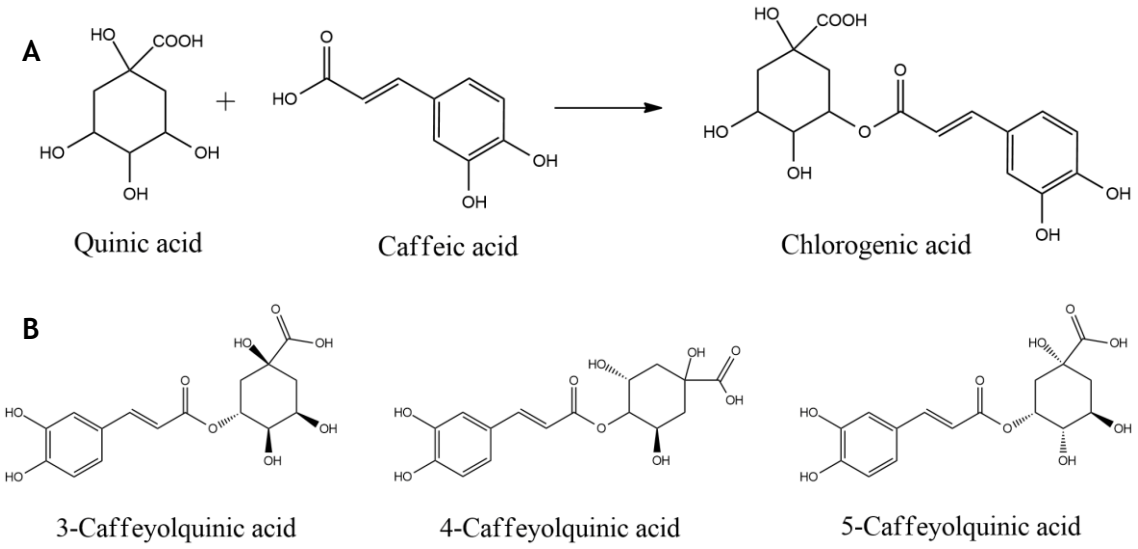


***Figure 1**. A) Reaction between quinic acid and caffeic acid resulting in the formation of chlorogenic acid. B) Most common isomers of chlorogenic acid.*

Green coffee is the richest source of CGA with the content of 4-14% (Perrone et al., 2008). In fact, high levels of chlorogenic acids have been measured in both coffee Arabica and Robusta, although greater values are present in Robusta. Chlorogenic acid is able to directly interact with reactive oxygen species (Kono et al., 1997), making it an effective OH• scavenger (Shi et al., 2006). Although the exact molecular mechanism underlying its antioxidant activity is unknown, it is attributed mainly to the double bond conjugated catechol structure of the phenyl ring (Nallamuthu, Devi, & Khanum, 2015). However, other numerous biological properties have been credited to this compound, namely: antimicrobial (Puupponen-Pimiä et al., 2001; Zhu, Zhang, & Lo, 2004), anti-obese (Cho et al., 2010), anti-inflammatory (Shan et al., 2009; Shin et al., 2015), neuroprotective (Bouayed, Rammal, Dicko, Younos, & Soulimani, 2007; Li et al., 2008), anti-diabetic (Karthikesan, Pari, & Menon, 2010), radio protective (Cinkilic et al., 2013), prevention of diseases associated with oxidative stress (namely cardiovascular, cancer and neurodegenerative) (Kasai, Fukada, Yamaizumi, Sugie, & Mori, 2000; K. Lee et al., 2012), slow the release of glucose into the bloodstream and also antiviral, hepatoprotective and immunostimulatory activity in vitro (Farah, 2012).

Caffeic acid, considered one of the main representative of the hydroxycinnamic and phenolic acids, also offers several beneficial properties, with particular highlight to its antioxidant activity. Other studies also attribute biological and pharmacological properties to caffeic acid (Jayanthi & Subash, 2010) namely: antioxidant (Gebhardt & Fausel, 1997), antiviral (Frank, Thiel, & MacLeod, 1989), anti-inflammatory (Joyeux, Lobstein, Anton, & Mortier, 1995), anticarcinogenic (Challis & Bartlett, 1975) and immunomodulatory activities (Iwahashi, Ishii, Sugata, & Kido, 1990).

2.1.1. Changes during coffee roasting

During roasting, green coffee beans undergo several changes (such as pyrolysis, Maillard reaction, Strecker degradation and caramelization) leading not only to the development of characteristic properties of the coffee beverage such as flavor, aroma and color but also to the formation of new compounds (Alves, Almeida, Casal, & Oliveira, 2010; Buffo & Cardelli-Freire, 2004; Esquivel & Jiménez, 2012). Due to chlorogenic acids thermal instability, roasting temperatures trigger several reactions, specifically isomerization, epimerization, lactonization, degradation to low-molecular-weight compounds such as phenols and cathecols (Figure 2), and to a lesser extent incorporation into melanoidins through noncovalent or covalent bounds (Bekedam, Schols, Van Boekel, & Smit, 2008; Farah, 2012; Ludwig et al., 2012; F. M. Nunes & Coimbra, 2009). Since chlorogenic acid shares the same building blocks as caffeic and quinic acids, these last two may also be incorporated into melanoidins. It should be borne in mind that roasting results in the loss of antioxidant activity of coffee brew and is also responsible for the production of harmful compounds such as carcinogenic compounds (e.g. polycyclic aromatic hydrocarbons, though in small amounts) (Orecchio, Ciotti, & Culotta, 2009) and acrylamide (right within the first minutes of the roasting process) (Esquivel & Jiménez, 2012). In order to avoid loss of bioactive compounds as well as the formation of dangerous compounds, coffee beans can also be used without roasting. Thus, green coffee beans are extracted with hot water (Suzuki, Kagawa, Ochiai, Tokimitsu, & Saito, 2002) ethanol (Thom, 2007) or their mixture (Naidu et al., 2008), producing “green coffee extracts” which may later be encapsulated, maintaining coffee beans benefits.

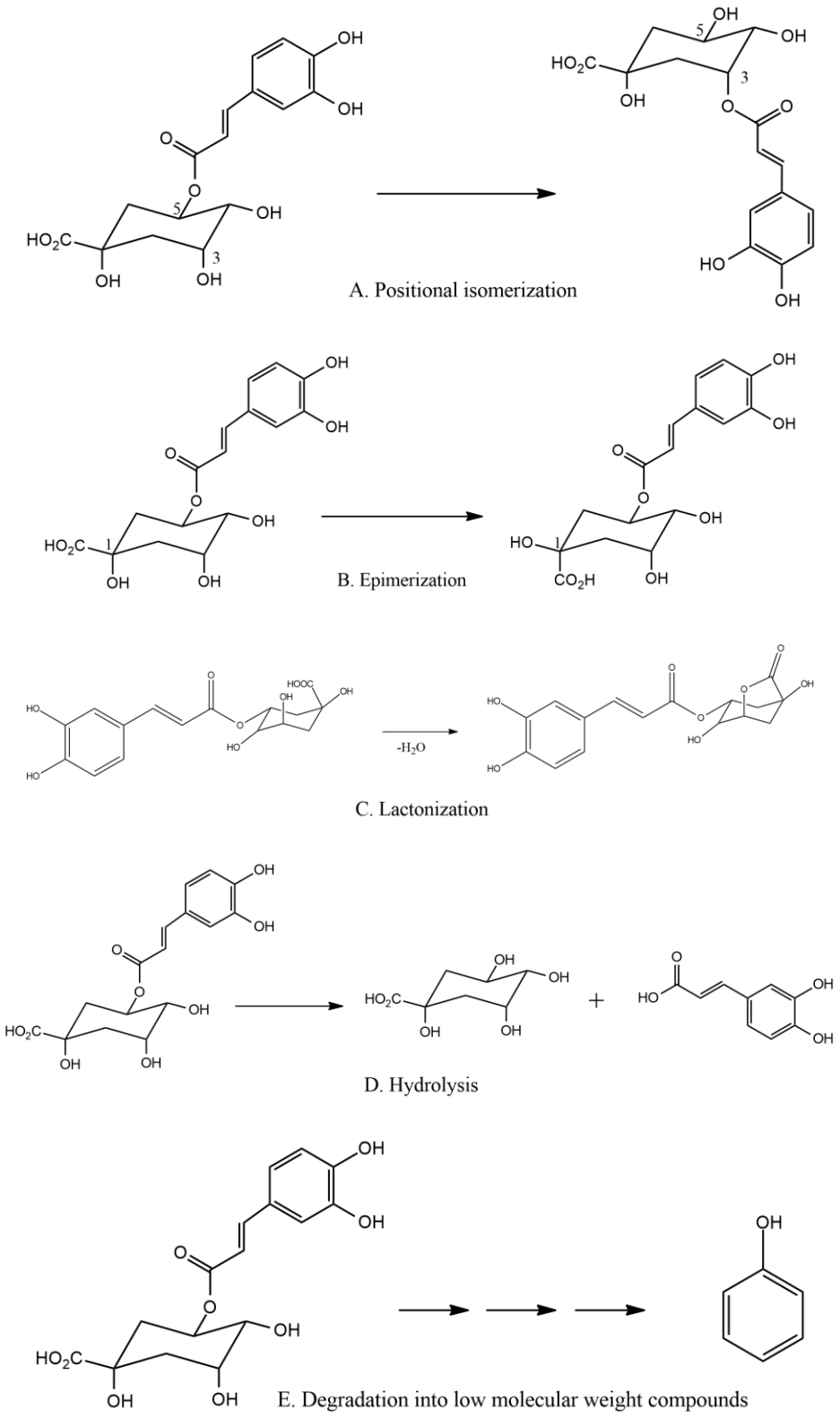


Figure 2. Reactions CGA suffers during coffee roasting.

2.2. Microencapsulation of antioxidants

Microencapsulation is a technique in which solid, liquid or gaseous particles (referred to as the core, internal phase or fill) are surrounded by a coating material (referred to as wall, shell, coating or membrane) creating a microcapsule with a range of diameters between a few micrometers and a few millimeters (Agnihotri, Mishra, Goda, & Arora, 2012; Dubey, Shami, & Bhasker Rao, 2009; P.Venkatesan, R.Manavalan, & K.Valliappan, 2009; Umer, Nigam, Tamboli, & Nainar, 2011).

Particles size may be classified as: macro (>5000 μm); micro (1.0–5000 μm); and nano (<1.0 μm) (Jafari, Assadpoor, He, & Bhandari, 2008). Particle size has great influence on food applications sensorial properties specially through its influence on texture. The presence of particles larger than 30 μm may induce a gritty mouthfeel sensation (Merkus & Meesters, 2013).

Regarding morphology, two major classifications are generally recognized: microcapsules (reservoir system), where the core is surrounded by an outside layer or microspheres (matrix type), where the core is homogenously integrated within the shell material (Zhongxiang Fang & Bhandari, 2010; Peres, 2011). Moreover, microparticles may present other morphologies such as mononuclear with multiples shells and microparticles clusters. Microparticles morphology depends on the core and wall material as well as on the technique engaged (Dubey et al., 2009; Jyothi & Seethadevi, 2012; Umer et al., 2011). Structure of microparticles can be assessed through techniques such as scanning electron microscopy (SEM) (Jafari, Assadpoor, He, et al., 2008). Examples of microparticles morphologies are shown in Figure 3.

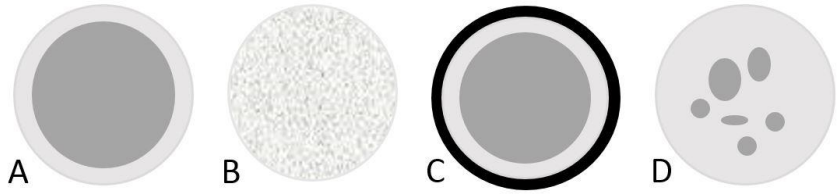


***Figure 3**. Microparticles different morphologies: A) microcapsule (reservoir type), B) microsphere (matrix type), C) capsule with two layers and D) capsule with several cores.*

Numerous reasons lead to the use of the microencapsulation techniques, mainly the following: increase stability by isolating active ingredients from its surroundings in order to prevent environment deterioration, delay evaporation of a volatile core, mask core undesired properties such as taste, odor or activity, controlled and/or targeted release of active ingredients, transform a liquid active ingredient into a dry solid structure and improve product esthetics and marketing perception, while maintaining core properties (Agnihotri et al., 2012; Dubey et al., 2009; Jyothi & Seethadevi, 2012; P.Venkatesan et al., 2009; Umer et al., 2011)

Several factors should be considered when using microencapsulation, namely: core material, microencapsulation technique, wall material, release mechanism and microparticles application.

The core composition may vary - a liquid core might include dispersed and/or dissolved constituents and a solid core can also be a combination of different constituents (active ingredients, stabilizers, diluents, recipients and release-rate retardants or accelerators) – allowing a flexible microcapsule development (Jyothi & Seethadevi, 2012; Umer et al., 2011).

2.2.1. Microencapsulation techniques

Some criteria should be considered when preparing the microparticles such as high core concentration incorporation; system stability with high shelf life; biocompatibility and particle size and distribution (Jyothi 2012; Umer et al. 2011; P.Venkatesan et al. 2009; Agnihotri et al. 2012). Technique selection for microencapsulation is complex and mainly dependent on physical and chemical properties of core and coating materials as well as the intended application of food ingredients (Poshadri & Kuna, 2010). There are various techniques available to choose from since no single encapsulation process is adaptable to all core materials or product application (Wilson & Shah, 2007). These techniques may be divided into chemical or mechanical processes though this classification can be variable and somewhat misleading. Table 2 shows some microencapsulation techniques used in the food industry, their classification and characteristics.

Table 2. Examples of microencapsulation techniques used in the food industry and characteristics.

Technique	Classification	Steps	Cost	Typical morphology
Spray-drying	Physical mechanical	1. Preparation and homogenization of feed dispersion 2. Atomization 3. Dehydration	Low	Matrix
Extrusion	Physical mechanical	1. Melting of the coating solution 2. Addition of core into coating solution 3. Passing of mixture through dehydrating liquid 4. Drying	High	Matrix
Liposome entrapment	Physical mechanical	1. Microfluidization 2. Ultra-sonication 3. Reverse-phase evaporation	High	Variable
Inclusion Complexation	Physical chemical	1. Mixing of compounds 2. Complexes formation through variable processes	Medium-low	Reservoir
Coacervation	Physical chemical	1. Preparation of a dispersion with three chemical immiscible phases 2. Coating deposition 3. Coating solidification 4. Use of cross-linker (optional)	High	Reservoir

Adapted from (Azeredo, 2005; Zhongxiang Fang & Bhandari, 2010; Gouin, 2004; Jyothi & Seethadevi, 2012; Poshadri & Kuna, 2010; Wilson & Shah, 2007; Zuidam & Shimoni, 2010)

2.2.1.1. Spray-Drying

Among the microencapsulation methods presented, spray-drying is the most used in the food industry due to several factors such as: process simplicity, relatively low cost, flexibility, allowing continuous operation, high stability of the final dried product (due to low moisture content), high volume reduction, ease of handling, transportation and storage of the particles. Also, it can be used for many heat-labile (low-boiling point) materials because of the lower temperatures the core material reaches. (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007; Nedovic et al., 2011; Peres, 2011; Sosnik & Seremeta, 2015). It is broadly used for large-scale encapsulation of several encapsulated substances, such as antibiotics, medical ingredients, additives, vitamins and polyphenols, among others (Nedovic et al., 2011; Peres, 2011). However, some limitations include high energy consumption, waste of supplied heat, high initial capital investment, low yields at laboratory scale and non-uniform conditions in the drying chamber (Nedovic et al., 2011; Sosnik & Seremeta, 2015).

Schematic pathway of the procedure is presented in Figure 4. It begins with preparation of the feed, which may be a solution, emulsion or suspension. After homogenization of the feed, this mixture is then fed into the spray dryer and atomized with a nozzle or spinning wheel. A spray is formed which is then dried by the inlet hot drying air. The resulting microparticles are transported to a cyclone and collected by falling on to the collector (Gharsallaoui et al., 2007; Phisut, 2012). Retention of the core material happens due to chemical and physical properties and interactions between core and coating materials, process temperature and also by the nature and the performance of the encapsulating support (emulsion stabilizing capabilities, film-forming ability and low viscosity at a high concentration). Spray-dried microparticles conventionally have matrix type morphology (Jafari, Assadpoor, Bhandari, & He, 2008).

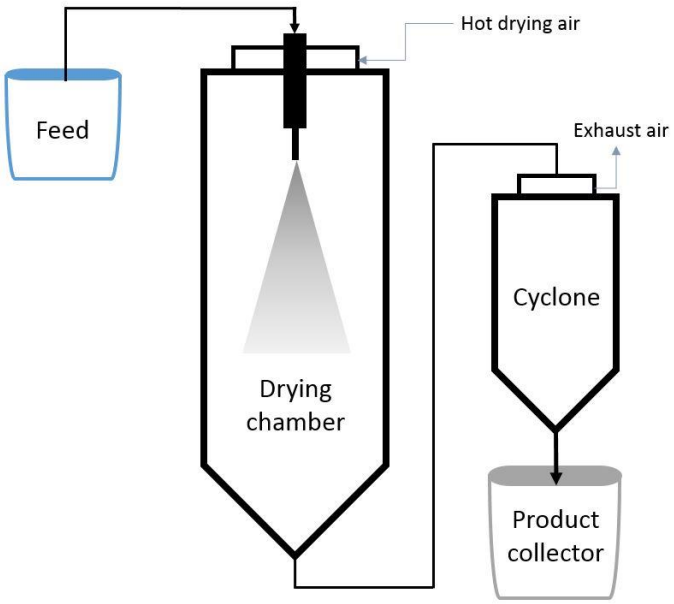


***Figure 4**. Spray-drying schematic pathway.*

There are differents ways to determine the capture efficiency of the microspheres also called percent of entrapment or entrapment efficiency (EE). One method is by allowing washed microspheres to lyse and then determine the ammount of active ingredient in the lysate (Equation 1). Other possilble method is to measure the residual ammount of active ingredient in the solution immediately after dispersion of the microparticles in the solvent and then subtract this value to the total loaded amount of active ingredient (Equation 2). This residual value correspondes to the ammount of active ingredient that was not withheld in the microparticles.

$$EE\ (\%) = \frac{active\ ingredient\ in\ the\ microspheres\ (lysate)}{active\ ingredient\ in\ feed\ solution} \times 100 \qquad \text{(Eq. 1)}$$

$$EE\ (\%) = \frac{active\ ingredient\ in\ feed\ solution - free\ residual\ active\ ingredient}{active\ ingredient\ in\ feed\ solution} \times 100 \qquad \text{(Eq. 2)}$$

In order to increase EE, some operating factors could be optimized such as feed temperature (it might change viscosity and therefore homogenization ability of the feed solution), air inlet temperature (low temperatures results in low evaporation rate while high temperatures might cause degradation of compounds), and air outlet temperatures (depends on the feed and air inlet temperature though it should be between 50 - 80 °C) (Peres, 2011). Nozzle geometry should also be considered when using this method (Z. Fang & Bhandari, 2012; Munin & Edwards-Lévy, 2011).

2.2.2. Encapsulating agents

The selection of the encapsulation method and wall materials is interdependent. The physical and chemical properties of the microparticles is also determined by the selected coating material. 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; Jyothi & Seethadevi, 2012; P.Venkatesan et al., 2009; Umer et al., 2011).

There are numerous substances available for microencapsulation by spray-drying. However, considerations must be made regarding their use in the food industry since some coating agents accepted for drug encapsulation are not adequate for food applications unless they are certified as "generally recognized as safe" (GRAS) materials (Nedovic et al., 2011). The most common wall materials used in the food industry can be divided in carbohydrates, proteins (whey proteins, caseinates, and gelatin) and new biopolymers (Jafari, Assadpoor, Bhandari, et al., 2008). Carbohydrates include modified and hydrolyzed starches (amylose, amylopectin, dextrins, maltodextrins, polydextrose, syrups), cellulose derivatives and cyclodextrins, plant exudates and extracts (such as gum Arabic, gum tragacanth, gum karaya, mesquite gum, galactomannans, pectins and soluble soybean polysaccharides), marine extracts (such as carregeenans and alginate) and microbial and animal polysaccharides (such as dextran, chitosan, xanthan and gellan) are also suitable for food applications. Most commonly used proteins consist of whey proteins, milk protein, gelatin and gluten (Nedovic et al., 2011). Table 3 summarizes some of the coating agents used for food applications by spray-drying technique, its main properties and applications.

Table 3. Some coating materials used for spray-dried particles, properties and examples of applications.

Coating category	Coating agents	Properties	Examples of applications
Carbohydrates			
Hydrolyzed Starches	Corn syrup Maltodextrin	Very good oxygen barrier Low viscosity at high solids Low cost	Citral and linalyl acetate; Cheese aroma; Linoleic acid; Orange peel oil; Lemon oil.
Modified Starches	Acetylated starch	Good emulsion stabilizer Low cost	Meat flavor; Fish oil; Orange oil; Black pepper oleoresin; Vitamin E.
Cyclodextrins	α-, β-, γ-, Cyclodextrins	Good inclusion of volatiles Great oxygen barrier Relatively expensive	Pine flavor; Shiitake flavor; Ethyl hexanoate; Caraway fruit oil; Lemon oil.
Gums	Arabic gum Xanthan Alginates	Good emulsion stabilizer Good viscosity and solubility Good retention of volatiles Might contain impurities Price dependent on availability but a little expensive	Orange peel oil; Cardamom oil; Vegetable oils; Linoleic acid; Soy oil; D-limonene; Ethyl butyrate.
Proteins			
Milk proteins	Whey proteins Caseinates	Good emulsion stabilizer Good solubility in water Properties depend on external factors (pH, ionic strength) Potential allergenic Relatively expensive	Milk fat; Linoleic acid; Soy oil;
Other proteins:	Soy proteins, Egg proteins, Gelatin Gluten		Fish oil, Soy oil, Oregano; Marjoram.
Other			
Other biopolymers	Chitosan, Gelatin	Variable properties Potential benefits to core stability, release mechanism or function	Orange oil; PUFAs; Fish oil.

Adapted from *(Z. Fang & Bhandari, 2012; Gharsallaoui et al., 2007; Jafari, Assadpoor, He, et al., 2008)*

2.2.2.1. Cellulose derivatives: carboxymethylcellulose

Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation (DeVries et al., 2001). Therefore, a high-fiber diet has many benefits including normalization of bowel movements, lower cholesterol levels, controlled blood sugar levels, healthy weight and lower risk of diabetes and heart disease. Dietary fiber is found mainly in fruits, vegetables, whole grains and legumes and can be classified as soluble fiber or insoluble fiber. The first type forms a gel-like structure when mixed with water and is able to be readily fermented by intestinal bacteria leading to lower blood cholesterol and glucose level as well as a modest increase in fecal bulk. On the other hand, insoluble fiber is not able to be fermented, resulting in maintenance of the fiber mass in the large bowel promoting material movement through the digestive system and, therefore, helping with constipation issues (Schneeman, 1987). Awareness of dietary fiber benefits acts as an extra incentive for food manufacturers to include these compounds into the final product (Cheung, 2013). Therefore, addition of dietary fiber into microparticles is of great interest since it increases the nutritional value of the food products where it is implemented. Constituents of dietary fiber are shown in Figure 5.

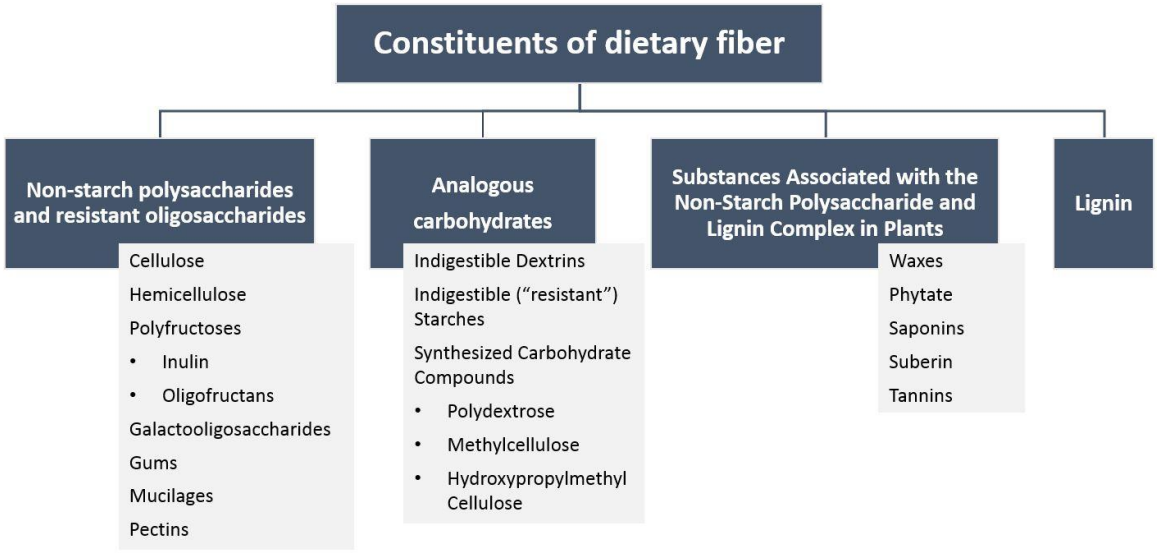


***Figure 5**. Constituents of dietary fiber. Adapted from (DeVries et al., 2001)*

Cellulose derivatives are dietary fibers capable of being used as coating agents in spray-drying techniques (Carmo, Fernandes, & Borges, 2015; Wandrey, Bartkowiak, & Harding, 2010). Cellulose is a non-digestible polymer of β-D-glucose: units linked by β-(1→4)-glycosidic bonds though the majority also contains a small percentage of other glucose units. Absence of side chains allows cellulose molecules to arrange together which promotes its insolubility in water and other ordinary solvents. Cellulose is widely available since it is the major structural material of plants. Natural sources include wood, cotton, flax, hemp, jute and straw though it might be synthesized by acetic acid bacteria or algae (DeVries et al., 2001).

Partial or complete reactions of the hydroxyl groups of cellulose may form cellulose derivatives suitable for food applications with different degrees of substitution (DS). Specific properties of these derivatives depend of factors such as the substituent type, its frequency, and distribution along the polymer backbone, the molar mass, and the molar mass distribution (Wandrey et al., 2010). Generally recognized as safe (GRAS) cellulose ethers are summarized in Table 4.

Table 4. Food grade (GRAS) cellulose derivate and characteristics.

Cellulose derivative	CAS	E number	DS[a] or DR[b]	Water solubility
Cellulose	9004-39-1	460	-	Insoluble
Methyl-	9004-67-5	461	DS 1.3-2.6	Soluble
Ethyl-	9004-57-3	462	DS 2.1-2.6	Insoluble
Hydroxypropryl-	9004-64-2	463	DR 4	Soluble in cold water (insoluble at T> 45 °C)
Hydroxypropylmethyl-	9004-65-3	464	DS (methyl) 0.9-1.8 DR (HP) 0.1-1.0	Soluble
Ehtylmethyl-	9004-69-7	465	-	Soluble in cold water
Na-Carboxymethyl-	9004-32-4	466	DS 0.4-1.4	Soluble in both cold and hot water

Adapted from (Wandrey et al., 2010).

[a]DS: Degree of substitution is the average number of substituted hydroxyl groups per saccharide unit;

[b]DR: Degree of reaction is the average number of reagent molecules reacted with one saccharide unit.

Na-CMC is a cellulose derivative that can be used as a suitable carrier for natural antioxidants due to its wide applications in food technology as the E number 466 due to its properties such as thickening, stabilizing and mouthfeel improving agent.

Sodium Carboxymethyl cellulose (Na-CMC)

Carboxymethyl cellulose (CMC), also known as cellulose gum, is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) bound to the hydroxyl groups of cellulose glucopyranose. It is produced by the alkali-catalyzed reaction of cellulose with chloroacetic acid (Figure 6) and is typically used as its salt form (normally combined with sodium). Its molar mass, chemical formula and other functional properties are variable since they depend on the degree of substitution and on the chain length of the cellulose structure. It usually presents itself as a white, tasteless, odorless and free-flowing powder, which is soluble in both cold and hot water, forming clear and colorless solutions. Solution viscosity has a great influence in its solubility. Viscosity values (1% aqueous solution) range from 25 mPa.s to 8000 mPa.s and it tends to decrease with temperature.

Since it is an anionic linear polyelectrolyte, CMC molecular conformation in aqueous solution is influenced by its solution concentration, ionic strength and pH. Therefore, CMC chains are most extended in solutions with low concentrations, low ionic strength and high pH; and usually coil in the reverse conditions. (Wandrey et al., 2010).

Na-CMC (sodium carboxymethyl cellulose) is a hydrophilic matrix-forming polymer with good swelling and erosion characteristics. On hydration, polymer chains of Na-CMC untangle forming a viscous layer around the dosage surface which is capable of controlling drug release. Main release mechanisms include diffusion of dissolved drugs across the gel layer and/or gel erosion. Contribution of each release system is influenced by drug solubility: diffusion is predominant for water-soluble drugs while erosion is a key factor for the release of poorly water-soluble molecules (Nokhodchi, Raja, Patel, & Asare-Addo, 2012; Palmer et al., 2011).

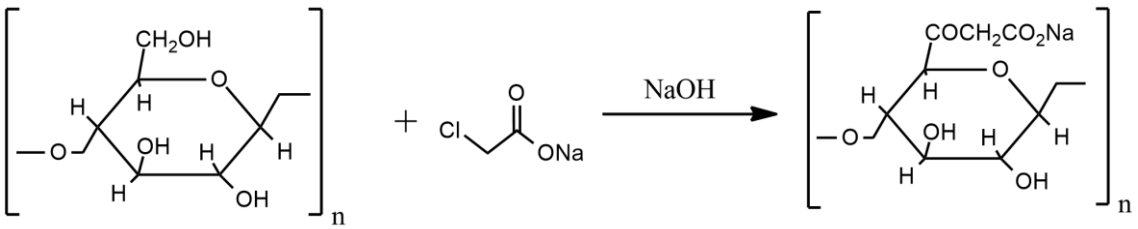


***Figure 6**. Reaction of cellulose with alkali and chloroacetic acid, producing sodium carboxymethyl cellulose.*

2.2.3. Controlled release

Controlled release is one of the main advantages of microencapsulation once it improves effectiveness and, consequently, decreases the need for higher doses of additives in the food industry (Azeredo, 2005). It might be classified as delayed, when the main goal is to delay compounds release until right time and/or place, or sustained, when the main goal is to control release rate. The actual release of compounds is often a combination of both types. Figure 7 exemplifies possible release profiles.

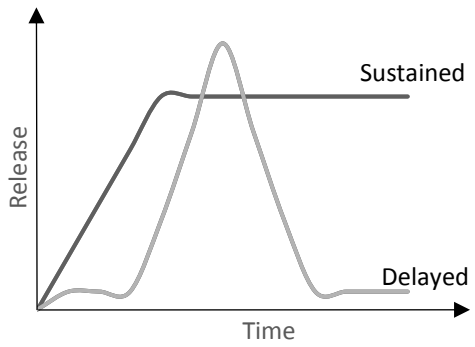


Figure 7. Generic representation of "sustained" and "delayed" release profiles.

Several factors may trigger the release though, typically, the main systems involved in the core release are shown in Figure 8. Core release may be caused or activated by: diffusion, degradation, solvent, pH, temperature and pressure (Azeredo, 2005).

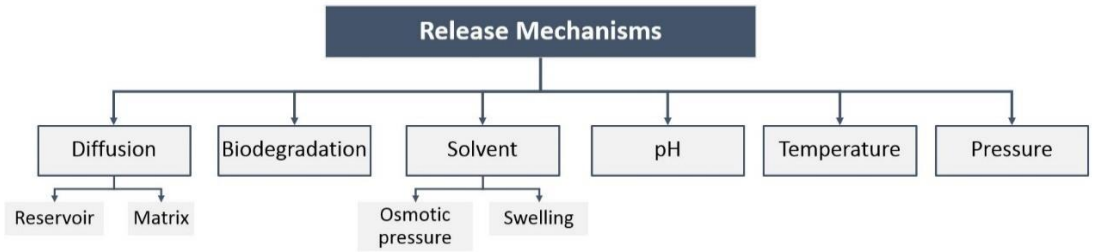


Figure 8. Diagram of a possible classification of release systems.

Diffusion-controlled systems may be divided in reservoir or matrix types. In the reservoir system, the core is able to slowly diffuse through a non-degradable layer coating. 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. Biodegradation systems involve the use of coating agents (e. g. proteins or lipids) capable of being degraded by enzymes. Solvent-activated release systems may be controlled by osmotic

pressure or swelling phenomena. Osmotic pressure occurs when there is a variation between the core concentration inside and outside of a semi-permeable membrane (only permeable to the solvent). The resulting pressure leads to a solvent flow to the inside of capsule, causing the exit of the saturated solution. On the other hand, swelling phenomena usually occur when the coating agent is a hydrogel matrix which is able to absorb great amounts of water before dissolving. Release rate is influenced by the water absorption rate of the coating. pH release systems occur due to changes in pH values that may lead to changes in the coating solubility. Temperature fluctuations may cause changes in the particles physical state and/or release rate resulting in the collapse or distension of the coating material (thermo-sensitive release) or in the fusion of the coating due to temperature rising (thermal-activated release). Pressure-activated release occurs when pressure is applied to the particles surface (e. g. chewing) (Azeredo, 2005). Figure 9 illustrates some release mechanisms based on diffusion, swelling and degradation systems.

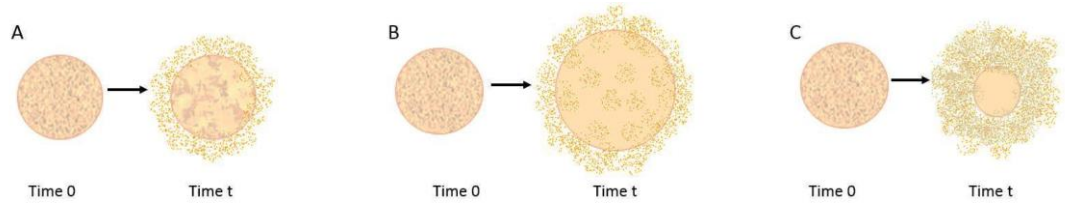


Figure 9. Common release systems: A. Diffusion, B. Swelling and C. Degradation.

The most common methods for controlled release in foods include solvent-activated release for hydrophilic encapsulates, and thermal release for fat particles (Wilson & Shah, 2007). However, pH control, addition of surfactants, enzymatic release, ultrasonics, grinding, and photo-release may also be release triggers in food products. Overall, particles type, geometry of the microparticles and core-wall interactions determine release profile. Microparticles resulting from atomization techniques usually have release mechanisms associated with solubility and diffusion processes (Azeredo, 2005). Conventional spray-dried microparticles tend to release their active agent immediately upon addition to water. Release might also depend on the porosity of the particles though addition of more hydrophobic and/or cross-linked carrier materials may result in a more gradual release upon dilution in water (Zuidam & Shimoni, 2010). Prediction of release profiles remains a challenge in biological systems, whether in food or in the gastrointestinal tract. However, identification of parameters as well as knowledge about mass transport and diffusion of the active compounds from a region of high concentration (microcapsule) to a region of low concentration (destination) facilitates the process. Controlled release patterns may be investigated using quantification methods to measure the compounds concentration released into a certain volume of solvent. Regarding antioxidants microencapsulation, its control release goal is to avoid an early and instant release, allowing a targeted and also prolonged effect.

CHAPTER 3: STATE OF THE ART

There is a wide number of studies regarding the microencapsulation of antioxidants, and coffee antioxidants are no exception. There seems to be a considerable attention to the microencapsulation of chlorogenic and caffeic acid, either as isolated compounds or combined in an extract. However, several studies use the aforesaid antioxidants from other sources besides coffee. Figure 10 shows some of the other natural sources chlorogenic and caffeic acid.

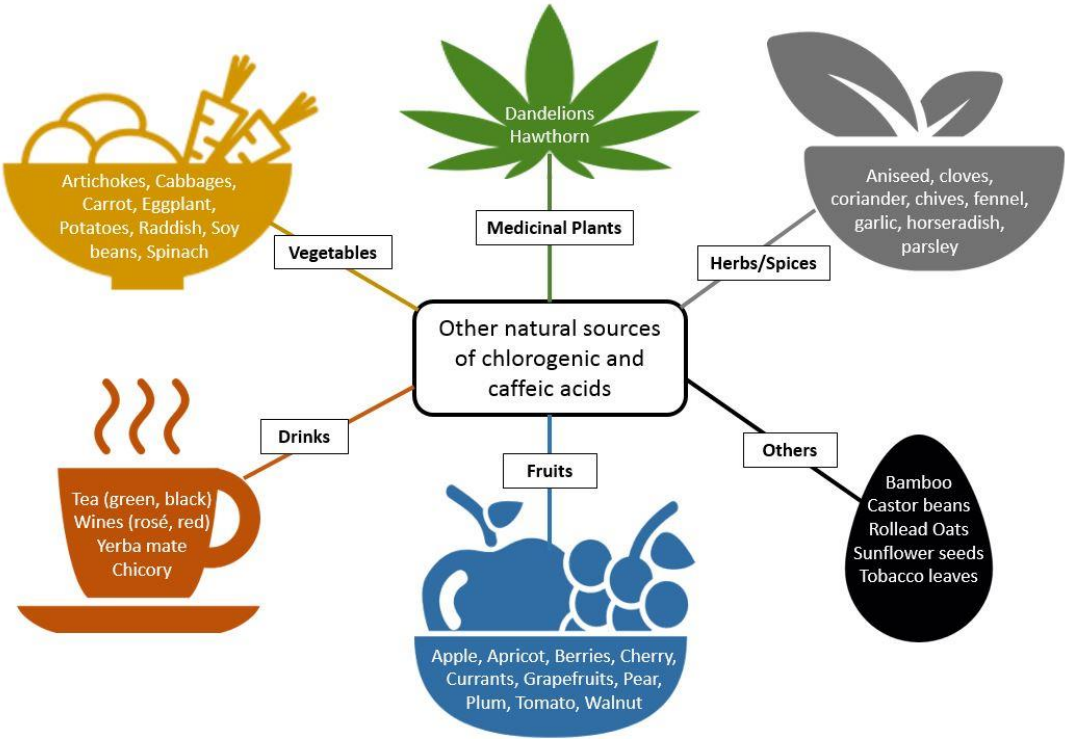


***Figure 10**. Some of the other natural sources (besides coffee) of chlorogenic and caffeic acid divided in the following categories: fruits, (plants used to produce) drinks/beverages, vegetables, medicinal plants, herbs/spices and others.*
[It should be borne in mind that not all of these sources contain both antioxidants, but at least one.]

The following Tables 5, 6, 7 and 8 compile information about recent publications studying the microencapsulation of coffee antioxidants (namely chlorogenic and caffeic acid). Due to their high amount, the found publications were distributed across different tables for a better and straightforward reading, with the following organization: Table 5 comprises the studies found about the microencapsulation of chlorogenic acid as a free compound, i.e., not combined in a mixture or extract; Table 6 comprises the studies found about the microencapsulation of caffeic acid as a free compound, i.e., not combined in a mixture or extract; Table 7 comprises the studies found about the microencapsulation of coffee extracts and Table 8 comprises the studies found

about the microencapsulation of chlorogenic and caffeic acids obtained from other natural sources other than coffee. Publications comprised on the aforementioned tables were selected considering articles published at 2005 or later, which included at least one assay about the microencapsulation of chlorogenic and caffeic acids. No further restrictions were made during article selection regarding the source of said compounds, encapsulating method or agent, characterization techniques nor about whether the compound was individually encapsulated or within an extract, mixture or combination. Tables 5 and 6 are organized first by method and then by the encapsulating agent used in each publication. Table 8 is firstly organized by the type of core (with the following order: 1. extracts containing only caffeic acid; 2. extracts containing only chlorogenic acid and 3. extracts containing both caffeic and chlorogenic acids) and then organized by method for each type of core.

The presented studies consisted predominantly in the preparation and characterization of microparticles varying the techniques and encapsulating agents (type, concentration, combination and formulation) used. Besides physicochemical characterization of the microparticles, release profiles/mechanism, antioxidant activity, storage stability and other properties were also investigated. Although the majority of the studies targeted the food industry for their products final application, some studies were performed considering final application in the cosmetic or healthcare industry. Figure 11 presents the main statistics on the publications featured on the aforementioned tables regarding the type of core, microencapsulation method and encapsulating agent used.

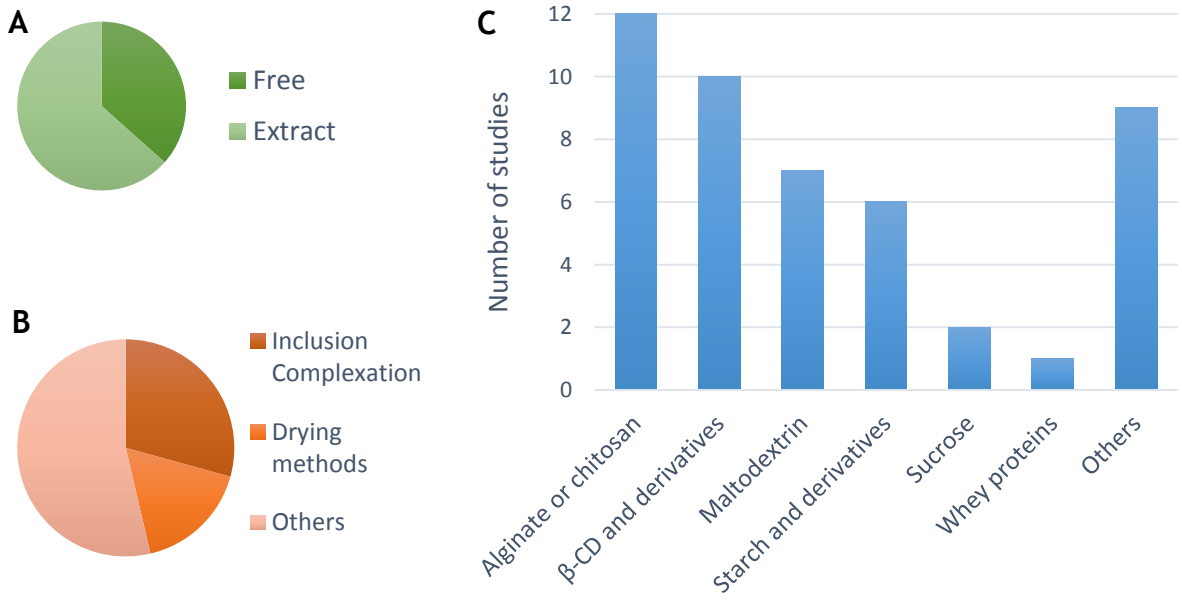


Figure 11. Main statistics on the found publications concerning microencapsulation of chlorogenic and caffeic acids regarding the A) type of core, B) microencapsulation method and C) encapsulating agent.

It is possible to see a strong prevalence of inclusion complexation as the microencapsulation method used probably due to its potential profitability and also due to the β-cyclodextrins versatility and availability (Brasileiro, 2011). Nevertheless, other microencapsulation methods are widely used, such as drying techniques (spray and freeze-drying) due to their simplicity, flexibility and easy scale-up. Other methods not so used in the found publications, though feasible and with good results, include liposome entrapment, yeast cell based microencapsulation, ionic gelation and co-crystallization. Regarding the type of core, there are more publications studying the encapsulation of chlorogenic and/or caffeic acid combined in an extract rather than in the free form. For wall materials, a wide variety seems to be used, though some common ones include alginate, chitosan, β-CD, maltodextrin, starch and derivatives, sucrose, whey proteins, among others. It should be borne in mind that some publications referred microencapsulation processes using more than one type of wall material.

The overall results of the studies show a fair entrapment efficiency, satisfactory release profiles, increased storage stability and bioavailability. In addition, antioxidants properties such as antioxidant activity and antimicrobial capacity were not compromised during the experiments.

Although microencapsulation proves to be effective in the protection and release of antioxidants, a few techniques have been investigated to enhance its benefits. The use of a coating or shell material around the particles was found to cause retardation of the bioactive release and even increase antioxidants properties (e.g. antioxidant activity) (Sabaghi, Maghsoudlou, & Khomeiri, 2015). The implementation of high hydrostatic pressure to the coating agent may also improve its properties. (Deladino, Teixeira, Navarro, Alvarez, & Martino, 2014) found that although native starch was able to transport both minerals and polyphenols, high hydrostatic pressure treatment increased starch ability to do so. The incorporation of a starch filler into an alginate matrix proved to be an easy, economical and functional way to optimize antioxidants protection and delivery into food products. Incorporation led to an increase in entrapment efficiency, release profile modulation and it also diminished the contribution of matrix erosion to the release mechanism (Córdoba, Deladino, & Martino, 2013; Miriam, 2013).

***Table 5**. Studies concerning the microencapsulation of chlorogenic acid (as an isolated compound), regarding the wall material, microencapsulation method, main objectives and results.*

Method	Wall	Objective	Results	Ref.
Inclusion complexation	β-cyclodextrin	Investigate the effects of CGA/β-CDs complexes on preservation of anthocyanins and color quality of grape juice.	Degradation of anthocyanins was reduced when inclusions were added to grape juice (CGA/HP-β-CD > CGA/β-CD complex > free CGA).	(Shao, Zhang, Fang, & Sun, 2014)
		Determine the effect of the complexation process on the antioxidant capacity of CGA.	Complexation process produced a slight increment in the antioxidant activity of CGA (between 7 and 14%).	(Alvarez-parrilla, Rosa, & Torres-, 2005)
		Develop and characterize β-CD complexes with CGA for industrial applications.	β-CD complexes improved CGA storage stability with no significant changes in antimicrobial activities.	(Zhao, Wang, Yang, & Tao, 2009)
	Hydroxypropyl-β-cyclodextrin	Investigate the inclusion complexation behavior of CGA with HP-β-CD, in both solution and the solid state.	HP-β-CD can be used for encapsulation and release of CGA since it formed a stable complex with higher antioxidant capacity than free CGA.	(Chao et al., 2011)
	Chitosan	Synthesize chitosan–CGA covalent complex and study its structural and functional properties.	Chitosan–CGA covalent complex was more thermally stable in terms of antioxidant activity and it increased chitosan viscosity.	(Z. Wei & Gao, 2016)
	Amylose	Study the use of amylose to form complexes by conventional hydrothermal method, using CGA and 4-O-palmitoyl CGA.	Both amylose–CGA and amylose–4-O-palmitoyl CGA are able to assembly. The latter forms amorphous complexes.	(Lorentz et al., 2012)
Ionic gelation	Chitosan	Prepare and characterize the CGA loaded chitosan nanoparticles with preserved antioxidant activity, controlled release property and enhanced bioavailability.	Synthesized nanoparticle with sustained release property can ease the fortification of food matrices targeted for health benefits through effective delivery of CGA in body.	(Nallamuthu et al., 2015)
Liposome entrapment	Soybean lecithin combined with cholesterol	Prepare, characterize and evaluate the bioavailability and *in vivo* antioxidant activity of CGA-loaded nanoparticles.	Findings present a liposomal formulation with significantly improved oral bioavailability and an increased *in vivo* antioxidant activity of CA.	(Feng et al., 2016)
Yeast-cell-based microencapsulation	Yeast cells	Microencapsulate CGA in yeast, study its characteristics and stability as a powder.	Capsules were stable and prevented CGA degradation without slowing down the release.	(Shi et al., 2006)
n. s.	Modified tapioca starch-filled calcium alginate hydrogel beads	Encapsulate and characterize the resulting beads as well as their release profile under simulated gastrointestinal conditions.	The use of the proposed system is a suitable and economical alternative for modulating physicochemical properties and release rates of bioactive compounds for food applications.	(Lozano-Vazquez et al., 2015)

n. s.: not specified

***Table 6**. Studies concerning the microencapsulation of caffeic acid (as an isolated compound), regarding the wall material, microencapsulation method, main objectives and results.*

Method	Wall	Objective	Results	Ref.
Inclusion complexation	Hydroxypropyl-cyclodextrin	Study of the complexation behavior and experimental conditions.	Best experimental conditions were: inclusion in acid solution and inclusion ratio of 1:1. Spatial configuration suggests CAF entrapped inside the hydrophobic core of HP-CD with the lipophilic aromatic ring and the portion of ethylene.	(Zhang, Li, Zhang, & Chao, 2009)
	β-cyclodextrin	Investigate the interaction between cinnamic acid derivatives and β-CD.	Experimental observations allowed the calculation of the binding orientations of β-CD with cinnamic acids.	(Liu et al., 2015)
Inclusion complexation followed by freeze-drying	Various β-cyclodextrins	Evaluate the effects of encapsulation with various CDs on some physicochemical and biological properties of seven polyphenols, prepared in solution or solid state.	Although a reduction in antifungal activity was observed, encapsulation in CDs improved aqueous solubility and photostability and reduced loss and degradation of polyphenols during use and storage.	(Kfoury et al., 2015)
Electrostatic layer by layer self-assembly technique	Dextran/chitosan	Encapsulate mixtures of polyphenols to achieve their controlled release.	Polysaccharide-based capsules can be successfully used to encapsulate and release low water-soluble molecules, such as polyphenols.	(Paini, Aliakbarian, Casazza, Perego, et al., 2015)
n. s.	Methoxy polyethylene glycol-b-poly(ε-caprolactone) copolymer	Produce caffeic acid phenethyl ester incorporated nanoparticles and study their antitumor activity against pulmonary metastasis model of CT26 colon carcinoma cells.	Resulting nanoparticles showed spherical shapes smaller than 300 nm and promising properties for antimetastatic chemotherapeutic. Caffeic acid phenethyl ester was continuously released from nanoparticles over 4 days.	(H. Lee et al., 2014)

n. s.: not specified.

Table 7. Studies concerning the microencapsulation coffee extracts regarding the wall material, microencapsulation method, main objectives and results.

Core	Method	Wall	Objective	Results	Ref.
Green coffee extract ▪ Chlorogenic acid ▪ Other hydroxycinnamic acids from green coffee	Inclusion complexation	β-cyclodextrin	Investigate the interaction between hydroxycinnamic and chlorogenic acids (free or included in β-CD) with different protein hydrolysates.	Encapsulating of the aforementioned acids in β-CD significantly increased their availability from processed food containing protein hydrolysates after digestion.	(Zaczy, Belica, & Pe, 2015)
Coffee brew ▪ Chlorogenic acid ▪ Caffeic acid			Deduce the molecular mechanisms of the formation of inclusion complexes of coffee-originated phenolic acids with beta-cyclodextrins.	The molecular modelling approach confirms experimental observations of the coffee-originated phenols abilities to form inclusion complexes with β-CD in aqueous environment.	(Górnas, Neunert, Baczyński, & Polewski, 2008)
Green coffee oil	Spray-drying	Combinations of modified starches or gum Arabic with maltodextrin (DE 10)	Assess the influence of different combinations of encapsulating agents on microencapsulation of green coffee oil by spray drying and two homogenization processes on emulsion preparation step.	The best condition for the microencapsulation of green coffee oil (using mixtures (75:25) of Hi-Cap starch/maltodextrin, emulsion homogenization at 50 MPa and air drying temperature of 170 °C) could also be applied for other food grade oil microencapsulation.	(Silva, Vieira, & Hubinger, 2014)
Roasted coffee oil ▪ Chlorogenic acid ▪ Caffeic acid	Miniemulsification solvent evaporation	poly (L-lactic acid) and poly(hydroxybutyrate-co-hydroxyvalerate)	Nanoencapsulation of roasted coffee oil and characterization of resulting nanocapsules by volatile compounds profile and investigation of oil recovery.	Total volatile concentration remained unchanged after encapsulation and the nanoparticles presented most of the sensorial compounds that are important to coffee aroma.	(Freiberger et al., 2015)

***Table 8**. Studies concerning the microencapsulation of chlorogenic and caffeic acids (from other natural sources besides coffee) regarding the wall material, microencapsulation method, main objectives and results.*

Core	Method	Wall	Objective	Results	Ref.
Satureja montana extract ▪ Caffeic acid	Spray-drying	Maltodextrin	Microencapsulate *S. montana* extract by spray-drying, study the influence of maltodextrin concentration and characterize resulting capsules.	Maltodextrin concentration showed influence on the content of beneficial health compounds and sensory evaluation of the microparticles.	(Vidović, Vladić, Vaštag, Zeković, & Popović, 2014)
Olive pomace extract ▪ Caffeic acid			Study the operative parameters and evaluate the microparticles.	Spray-drying forms olive pomace extract microparticles with good stability and antioxidant properties.	(Paini, Aliakbarian, Casazza, Lagazzo, et al., 2015)
Red wine *Cabernet sauvignon* ▪ Caffeic acid	Freeze-drying	9% (w/w) maltodextrin (DE 10) and gum Arabic	Investigate the stability of red wine phenolics encapsulated in a lower concentration of wall material (9%).	Water activity affected phenolics stability during storage. Antioxidant activity of the wine powder remained constant over 145 days at accelerated storage conditions.	(Rocha-parra, Lanari, Zamora, & Chirife, 2016)
Achillea millefolium extract ▪ Chlorogenic acid	Spray-drying	Maltodextrin	Solid–liquid extraction followed by spray drying of *A. millefolium* by-product from food industry.	Powders had adequate physical-chemical characteristics and moisture content for further used in food applications.	(Vladić et al., 2015)
Green tea extract ▪ Chlorogenic acid	n. s.	Chitosan	Evaluate the effect of chitosan incorporating green tea extract as an edible coating on the properties of walnut kernels.	Coating solution with 10 $g.L^{-1}$ chitosan and 5 $g.L^{-1}$ green tea extract was the best at reducing oxidation activity, fungal growth, and undesirable sensory properties.	(Sabaghi et al., 2015)
Dandelion (*Taraxacum officinale L.*) extract ▪ Chlorogenic acid (and ß-carotene)	n. s.	Alginate and pectin beads (combined with whey proteins)	Develop a technique to microencapsulate compounds from dandelion extract and characterize resulting beads.	Maximum encapsulation efficiency, while maintaining morphological properties was obtained using whey proteins combined with alginate.	(Belščak-Cvitanović et al., 2016)
Star fruit (*Averrhoa carambola*) pomace extract ▪ Chlorogenic acid ▪ Caffeic acid	Spray and freeze-drying	Maltodextrin (DE 20)	Determine optimum conditions for extraction and microencapsulation of polyphenols with maltodextrin by spray and freeze-drying.	Microcapsules from both methods can be incorporated in food systems to enhance their antioxidant property, though the ones resulting from freeze-drying showed higher encapsulating efficiency (78-97%).	(Saikia, Mahnot, & Mahanta, 2014)

Core	Method	Wall	Objective	Results	Ref.
Hypericum perforatum (St John's wort) extract ▪ Chlorogenic acid ▪ Caffeic acid (low amount)	Inclusion complexation	β-cyclodextrin	Study the inclusion behavior oh St John's wort extract in β-CD and complex characteristics.	Encapsulation in β-CD improves the thermal stability of nutraceutical antioxidants present in St John wort extract.	(Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, & Makris, 2010)
Different plant extracts ▪ Chlorogenic acid (in one plant) ▪ Caffeic acid	Extrusion	Alginate	Prepare, characterize and optimize medicinal plants extracts properties using encapsulation techniques and alginate as wall material.	Encapsulation could potentiate the action of the extract by promoting a protective effect and a sustained release of active constituents.	(Rijo et al., 2014)
Raspberry leaf, hawthorn, ground ivy, yarrow, nettle and olive leaf extracts ▪ Caffeic acid ▪ Chlorogenic acid	Electrostatic extrusion	Alginate-chitosan (with ascorbic acid for dissolution of chitosan)	Characterize the original and encapsulated plant extracts.	High encapsulation efficiency (80-89%) was obtained for all capsules. Although refrigerated storage reduces antioxidant stability, capsules biological activity is still high for food applications.	(Belscak-Cvitanovic et al., 2011)
Helichrysum Stoechas hydroalcoholic extract ▪ Caffeic acid ▪ Chlorogenic acid	Double emulsion-evaporation	Polycaprolactone	Characterize, microencapsulate and incorporate *H. Stoechas* into a moisturizer.	*H. Stoechas* demonstrates antioxidant potential and viability for microencapsulation.	(Barroso et al., 2014)
Sage and savory extracts ▪ Chlorogenic acid ▪ Caffeic acid	Solid lipid nanoparticles (SLN)	Witepsol and carnauba waxes	Develop and characterize SLN, and study their stability and phenolic compound when exposed to a simulated gastrointestinal tract.	Witepsol SLN were the ones that best maintained their physical integrity during digestion, showing to be the most stable vehicles for sage and savory extracts.	(Campos, Raquel, Sarmento, Maria, & Manuela, 2015)
Yerba mate extract (*Ilex paraguariensis*) ▪ Chlorogenic acid ▪ Caffeic acid	Co--crystallization	Sucrose	Evaluate the resulting co-crystallized products.	Powders were free flowing, stable, with reduced hygroscopicity but some solubility.	(Lorena, S, S, & N, 2006)
			Characterize resulting powders.	Powders showed high entrapment yield, maintaining antioxidant activity and other desirable properties.	(López-córdoba, Deladino, Agudelo-mesa, & Martino, 2013)

Core	Method	Wall	Objective	Results	Ref.
	Spray-drying	Maltodextrin	Microencapsulate yerba mate extract by spray-drying, study the influence of maltodextrin concentration and characterize resulting capsules.	Maltodextrin concentration influenced microcapsules properties, promoting polyphenol preservation during the spray drying process and stability during storage.	(G. L. Nunes et al., 2014)
	Ionic gelation	Calcium alginate (with corn starch as filler agent)	Microencapsulation and characterization of yerba mate extracts, and study of their release in simulated digestive fluids.	The addition of corn starch granules into calcium alginate capsules was an easy, economical and functional approach to optimize the antioxidant protection and delivery into food products.	(Miriam, 2013)
	n. s.	Corn starch calcium alginate	Produce and analyze a system for the simultaneous carrying of zinc sulfate and yerba mate polyphenols.	Proposed method was able to transport antioxidants and zinc with high antioxidant activity, maintaining morphological aspect.	(López-córdoba, Deladino, & Martino, 2014)
		Corn starch treated with high hydrostatic pressure	Test the ability of corn starch treated by high hydrostatic pressure to bind and carry zinc and magnesium salts and yerba mate extracts.	Although native starch was also able to transport both minerals and polyphenols, high hydrostatic pressure treatment increased starch ability to do so.	(Teixeira, Navarro, Martino, & Deladino, 2015)
		Corn starch treated with high hydrostatic pressure	Test the ability of corn starch treated with high hydrostatic pressure to bind and carry a yerba mate extract.	Corn starch was suitable as a carrier for the extract, maintaining its antioxidant power without affecting the polyphenol profile.	(López-córdoba et al., 2014)
		Calcium-alginate hydrogels (containing corn starch as filler)	Study the influence of corn starch granules incorporation (as a filling agent) into calcium alginate hydrogels in yerba extract beads.	Incorporation of the starch filler to alginate matrix increased the entrapment capacity, modulated the antioxidants release rate and diminished the contribution of matrix erosion to the release mechanism.	(Córdoba et al., 2013)
		Calcium alginate and calcium alginate-chitosan	Encapsulate and analyze lyophilized yerba mate extracts in calcium alginate beads (with and without a chitosan layer).	Although entrapment in chitosan coated beads was lower than control, encapsulation in alginate and in alginate–chitosan beads suitable for food supplementation.	(Lorena Deladino, Anbinder, & Martino, 2007)

n. s.: not specified

CHAPTER 4: MATERIALS AND METHODS

4.1. Materials

4.1.1. Reagents

Chlorogenic acid standard (3-caffeoylquinic acid, Ref. C3878-1G, $C_{16}H_{18}O_9$, CAS: 327-97-9, ≥ 95% purity) and caffeic acid standard (Ref. C0625-2G, $C_9H_8O_4$, CAS 331-39-5, ≥ 98% purity) were acquired from Sigma-Aldrich Chemical Co. (MO, USA). Carboxymethylcellulose sodium salt (Viscosity 830 mPa.s (25 ºC, 2% water), CAS: 9004-32-4) was obtained from VWR International (Haasrode, Belgium) and methanol (CAS: 67-56-1) from VWR International (Fontenay-sous-Bois, France). ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), CAS: 30931-67-0) was purchased from AppliChem GmbH (Darmstadt, Germany), potassium persulfate (CAS: 7727-21-1) was acquired from Panreac química (Barcelona, Spain) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, CAS: 53188-07-1, 97% purity) was obtained from Sigma-Aldrich Chemical Co. (MO, USA). Ethanol (96% purity) was obtained from AGA (Prior Velho, Portugal) and HCl (Hydrochloric acid, CAS: 7647-01-0, 37% purity) was purchased from Sigma-Aldrich Chemical Co. (MO, USA). Water was deionized in the laboratory using MilliporeTM water purification equipment (Massachusetts, USA). All the reagents used were of analytical grade purity.

4.1.2. Equipment

Weight measurements were performed with an analytical scale Mettler Toledo AG245 (Columbus, OH, USA). Quantification analysis of the antioxidants was accomplished using a spectrophotometer UV-Vis V-530 (Jasco), as well as the antioxidant activity assays. Microencapsulation was performed using a BÜCHI B-290 spray dryer (Flawil, Switzerland) with a standard 0.5 mm nozzle. pH measurements were achieved using a 900 Multiparameter Water Quality Meter (A & E Lab; Guangzhou, China). Particle morphology was assessed by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M) and particle size distribution was evaluated by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA).

4.2. Methods

4.2.1. Validation of analytical method for antioxidant quantification

Validation of analytical method was performed as described by (Gonçalves et al., 2015). Although High Performance Liquid Chromatography (HPLC) is a common and more sensitive method used for the quantification of CGA and CAF, previous studies show that HPLC columns might clog with some microparticles. Therefore, UV-Vis spectrophotometry was selected for the quantitative analysis of chlorogenic and caffeic acid using two high precision quartz cuvettes (Hellma Analytics) in a Jasco V-530 UV-Vis Spectrophotometer. Calibration standard solutions were prepared: 9 standards for chlorogenic acid (from 0.5 to 14 $mg.L^{-1}$) and 11 standards for caffeic acid (from 0.25 to 15 $mg.L^{-1}$). In order to obtain a linear response, all standard solutions presented absorbance values ≤ 1 AU. Absorbance reading was performed at room temperature (20 – 25 ºC), at maximum wavelength (323 nm for chlorogenic acid and 313 nm for caffeic acid), previously determined through the performance of an absorption spectrum. In order to be validated, a calibration curve was required to comply with the following parameters: having at least 5 different standard solutions; linearity range factor greater than 10; $R > 0.995$; $\frac{s_a}{a} \times 100 \leq 5\%$ and $b - ts_b < 0 < b + ts_b$ (where R represents the linearity coefficient, a the sensitivity, b the intercept of the regression and s_a and s_b as the corresponding standard deviations). Validation performance parameters such as repeatability, intermediate precision and accuracy as well as the limits of detection (LOD) and of quantification (LOQ) were also determined.

4.2.2. Preparation of microparticles by spray-drying

For the microencapsulation process, both required solutions were prepared the day before: the solution containing the core (chlorogenic or caffeic acid) and the solution containing the encapsulating agent (sodium carboxymethyl cellulose). Encapsulating agent solution (10 $g.L^{-1}$) was prepared by weighing 2.5 g of Na-CMC into 250 mL of deionized water, under stirring (500 rpm) at room temperature (20 – 25 ºC). Chlorogenic acid solution (10 $g.L^{-1}$) was prepared by weighing 52.6 mg of chlorogenic acid standard (3-caffeoylquinic acid, $C_{16}H_{18}O_9$, CAS: 327-97-9, ≥ 95% purity) into 5 mL of deionized water, under stirring (500 rpm) at room temperature (20 – 25 ºC). Caffeic acid solution (10 $g.L^{-1}$) was prepared by weighing 51.0 mg of caffeic acid ($C_9H_8O_4$, CAS 331-39-5, ≥ 98% purity) into 5 mL of deionized water, under stirring (500 rpm) at room temperature (20 – 25 ºC). Encapsulating agent solution was kept under stirring at room temperature (20 – 25 ºC) to ensure full saturation of the polymer and solutions of chlorogenic and caffeic acid were kept overnight sealed with parafilm at 4 ºC for stabilization. Approximately 30

min before the spray-drying process, 250 mL of the Na-CMC solution were mixed with 5 mL of the antioxidant solution, at room temperature (20 – 25 ºC). Therefore, the mass ratio of core to encapsulating agent was 1:50 (w/w) and the final concentration of the core in the feeding solution was 2% (w/w).

Microencapsulation by spray-drying was performed using a spray-dryer BÜCHI B-290 (Flawil, Switzerland) with a standard 0.5 mm nozzle. Experimental conditions for all samples (Table 9) were previously optimized by (Estevinho, Damas, Martins, & Rocha, 2014). The outlet temperature (T_{out}) is not an established condition but a result of the experiment and was around 63 ºC. Afterwards, the resulting microparticles/dried powder were collected and stored in falcon tubes sealed with parafilm and covered with aluminum foil at 4ºC until further analysis. Resulting microparticles included Na-CMC microparticles loaded with CGA, Na-CMC microparticles loaded with CAF and also non-loaded Na-CMC microparticles.

Table 9. Operating conditions for the microencapsulation process by spray-drying.

Operating conditions	Values
Feed flowrate (%)	15
T_{in}: Inlet Temperature (ºC)	115
T_{out}: Outlet Temperature (ºC)	63
Aspiration (%)	100
Pressure (bar)	6
Nozzle (mm)	0.5

4.2.2.1. Product yield

Product yield (PY) was calculated for all the experiments as the ratio between the mass of the output powder obtained at the end of the spray-drying process and the initial solid content of the feed solution, i.e., mixture between encapsulating agent (Na-CMC) and core (chlorogenic or caffeic acid). PY was calculated according to the following equation:

$$PY\ (\%) = \frac{mass\ of\ output\ powder}{solid\ content\ of\ feed\ solution} \times 100 \qquad (Eq.3)$$

4.2.3. Microparticles characterization

Microparticles were characterized by its particle size distribution and morphology.

4.2.3.1. 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 powder was suspended in ethanol before measurement. Samples were characterized by number and volume as an average of two runs of 60 seconds. Particle size distribution was performed for non-loaded Na-CMC microparticles, Na-CMC microparticles loaded with CGA and Na-CMC microparticles loaded with CAF.

4.2.3.2. Particle morphology

Particles morphology was assessed by SEM analysis using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M. Samples were coated with Au/Pd thin film for 100 seconds and with a 15 mA current, by sputtering, using the SPI Module Sputter Coater equipment. Morphology was assessed for Na-CMC microparticles loaded with CGA and Na-CMC microparticles loaded with CAF.

4.2.4. Controlled release studies and entrapment efficiency

The release profile of both antioxidants (chlorogenic and caffeic acids) encapsulated with Na-CMC was investigated under aqueous solutions with two different pH values: 5.6 (pH of normal deionized water) and pH 2. In order to reduce water pH to 2, HCl 37% was added to deionized water under stirring. Assays were performed by adding 3 mg of microparticles into 4.5 mL of water solution, at room temperature (20 – 25 ºC), protected from light and under continuous stirring with a small magnet. Duplicate and separate samples were performed and examined for each defined time interval: 0, 1, 2, 5, 10, 20, 30 and 45 minutes, followed by 1, 2, 4, 6 and 24 hours. Release profiles were obtained by measuring the amount of free core (chlorogenic and caffeic acid) in the samples which was released from the microparticles after the defined time interval. Quantification of the core compound was performed using the previously validated UV-Vis spectrophotometry method (Consult section 4.2.1.).

Entrapment efficiency (EE) was measured for all the experiments as the ratio between the amount of core (chlorogenic or caffeic acid) inside the microparticles and the total amount of antioxidant. To determine the amount of core trapped within the microparticles, the amount of core right after

dispersion in water, (which corresponds to the amount of core not trapped within the microparticles) was measured and subtracted to the total amount. The amount of core right after dispersion corresponds to the controlled release value at time zero. Therefore, the entrapment efficiency was measured using the following equation:

$$EE = \frac{Total\ amount\ of\ core - amount\ of\ core\ right\ after\ dispersion}{Total\ amount\ of\ core} \qquad (Eq.2)$$

4.2.5. Antioxidant Activity Assessment

The antioxidant activity was estimated using the ABTS radical scavenging assay, as described by (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). An aqueous stock solution of 7.4 mM ABTS (Sigma-Aldrich) was mixed with an aqueous stock solution of 2.6 mM of potassium persulfate, in equal proportions and allowed to react for 12 h at room temperature (20 - 25 ºC) in the dark. A small volume of the resulting solution was mixed with about 60 mL of methanol in order to obtain an absorbance of 1.10 ± 0.02 AU at 734 nm. Afterwards, 150 µL of the samples were mixed with 2850 µL of the prepared solution and allowed to react for 2 h in the dark before measuring the absorbance at 734 nm Jasco V-530 UV-Vis Spectrophotometer). Antioxidant activity was expressed in µM Trolox (Sigma-Aldrich) equivalents (TE), a synthetic analogue of vitamin E commonly used as antioxidant reference. The standard curve was linear between 10 and 380 µM. (Appendix B). Samples consisted of free antioxidants standards in solution (for both caffeic and chlorogenic acid) and microparticles at maximum release (after 4 h in aqueous solution) loaded with caffeic and chlorogenic acid and also non-loaded microparticles of carboxymethylcellulose.

4.2.6. Waste Management

During this work, the majority of the waste produced consisted of aqueous solutions containing sodium carboxymethylcellulose and trace amounts of chlorogenic acid and caffeic acid. Other waste generated included residues of methanol, ABTS, trolox and potassium persulfate from the antioxidant activity assessment. All waste was stored in a proper labelled bottle container until further treatment by the specialized waste management unit at the university: Environmental Management System of FEUP – EcoFEUP.

CHAPTER 5: RESULTS AND DISCUSSION

First part of the current work consisted in the validation of the analytical method. In this case UV-Vis spectrophotometry was used to quantify both chlorogenic and caffeic acid. This validation was done to ensure the method suitability for the quantification of both compounds and to prove that it can produce accurate and precise measurements within the scope of its intended use (i.e. to generate a release profile of the antioxidants from the microparticles over time). Main results of the validation of the analytical method for both antioxidants are presented in section 5.1. Additional information can be consulted in Appendix A.

Second part consisted in the production of microparticles of chlorogenic or caffeic acid by spray-drying using carboxymethylcellulose sodium salt as encapsulating agent. Results concerning the product yield are presented in section 5.2.

After encapsulation by spray-drying, characterization of the aforementioned particles was required in order to evaluate the process regarding application of the microparticles for food products. Results of microparticles characterization (regarding its size distribution and morphology) are presented in section 5.3.

Controlled release studies were also investigated considering the desired environment for microparticles release: digestive tract. Therefore, these experiments were performed in two different mediums: deionized water at pH 5.6 and pH 2. Release at pH 2 was tested in order to better simulate the acidic conditions experienced in the stomach. Through the release profiles, it was possible to estimate an associated entrapment efficiency by measuring the free antioxidant amount at time zero*. Controlled release profiles and obtained entrapment efficiency values are presented in section 5.4.

Antioxidant activity was also assessed in order to investigate whether the evaluated compounds maintain their antioxidant activity after release on the environment upon which they are intended to act. Results from this assay are presented in section 5.5.

* "Time zero" designation is merely representative due to the fact that measurements at exactly time zero would be hard to achieve.

5.1. Validation of analytical method for antioxidant quantification

CGA calibration curve was linear from 0.50 to 14 $mg.L^{-1}$ and the linear regression was the following: Abs (AU) = 0.0554 ± 0.0002 [CGA] ($mg.L^{-1}$) - 0.003 ± 0.002 (R^2 = 1.000). Values obtained for LOD and LOQ were, respectively, 0.10 and 0.34 $mg.L^{-1}$. Repeatability, intermediate precision and accuracy were determined using three standards solutions (1.0, 6.0 and 14 $mg.L^{-1}$). For each respective solution, repeatability values were 1.4%, 0.1% and 0.1%; intermediate precision values were 22%, 4.5% and 1.3% and accuracy values were 77.3%, 97.5% and 99.2%.

CAF calibration curve was linear from 0.25 to 15 $mg.L^{-1}$ and the linear regression was the following: Abs (AU) = 0.067 ± 0.001 [CAF] ($mg.L^{-1}$) + 0.009 ± 0.006 (R^2 = 0.999). Values obtained for LOD and LOQ were, respectively, 0.29 and 0.96 $mg.L^{-1}$. Repeatability, intermediate precision and accuracy were determined using three standards solutions (1.0, 6.0 and 12 $mg.L^{-1}$). For each respective solution, repeatability values were 0.6%, 0.1% and 0.1%; intermediate precision values were 16%, 3.0% and 1.9% and accuracy values were 86.6%, 96.5% and 102%.

Overall, the presented methods complied with the required constraints, are precise and accurate and are, therefore, suitable for CGA and CAF quantification in aqueous solutions. Additional information and results may be consulted in Appendix A.

5.2. Preparation of microparticles by spray-drying

5.2.1. Product yield

Product yield is one of the key parameters measured when evaluating a spray-drying process. Table 10 displays the PY values obtained for non-loaded carboxymethylcellulose microparticles (Na-CMC only) carboxymethyl cellulose microparticles loaded with chlorogenic acid (Na-CMC + CGA) and carboxymethyl cellulose microparticles loaded with caffeic acid (Na-CMC + CAF).

Table 10. Product yield results.

Microparticles formulation	Product Yield (%)
Na-CMC only	47.5
Na-CMC + CGA	37.6
Na-CMC + CAF	40.8

The product yield obtained (ratio between the mass of the output powder obtained at the end of the spray-drying process and the initial solid content of the feed solution including both Na-CMC and antioxidant mass) was similar between microparticles loaded with CGA (37.6%) and microparticles loaded with CAF (40.8%). These values are quite satisfactory considering the employed method and scale used. The fact that non-loaded microparticles obtained higher yield (47.5%) suggests that the presence of antioxidant in the feeding solution may detrimentally interfere with the drying process. However, the difference is not significant.

Losses of the solid content may be explained by the significant small amount of used raw materials when compared to the equipment dimensions. Particles size may also contribute to solid losses since small microparticles are suctioned by the vacuum filter and therefore fail to be collected in the final powder. Similar results were found by (Estevinho et al., 2014) after producing microparticles containing β-galactosidase, using different biopolymers, obtaining product yields ranging from 36 to 59% (56% for arabic gum, 48% for chitosan, 59% for modified chitosan, 36% for calcium alginate and 37% for sodium alginate). Also, (Sansone et al., 2012) encapsulated soybean extracts with sodium carboxymethyl cellulose, obtaining a product yield of 65.3%, while (Su et al., 2007) obtained yield values ranging from 53-62% when microencapsulating *R. salvia miltiorrhiza* using a mixture of gelatin and sodium carboxymethyl cellulose with different spray-drying conditions.

5.3. Microparticles characterization

5.3.1. Particle size distribution

Particle size is a relevant parameter when considering food applications. Larger particles may change product texture or cause gritty sensation in the mouth. On the other hand, particle size and size distribution can also have an effect on microparticles release profile. The size distribution of the microparticles was evaluated by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyzer. Table 11 shows microparticles mean diameter considering both volume and number distribution. Figure 12 shows particle size distribution by volume and number.

Table 11. Mean diameters obtained from particle size distribution, considering volume and number distribution.

Samples	Mean diameter (μm)	
	Differential volume	Differential number
Na-CMC only	13.49	0.16
CMC + CGA	8.37	0.44
CMC + CAF	8.78	0.41

Considering volume distribution, the mean diameter of non-loaded Na-CMC microparticles (13.6 μm) was higher than the mean size of Na-CMC loaded microparticles. However, microparticles loaded with CGA and CAF showed similar mean size values (8.37 and 8.78 μm, respectively). Number distribution also shows similar mean diameters for microparticles loaded with CGA (0.44 μm) and CAF (0.41 μm) but non-loaded Na-CMC microparticles show smaller mean diameter size (0.16 μm). Results therefore suggest that the presence of these antioxidants influenced the mean size of the microparticles in a similar way.

Particles with diameters smaller than 20.64 and 21.97 μm are responsible for 90% of the total volume of microparticles loaded with CGA and CAF, respectively. On the other hand, particles with diameters smaller than 1.64 and 1.56 μm are responsible for 90% of the total number of microparticles loaded with CG and CAF, respectively.

Volume distribution of non-loaded Na-CMC microparticles presents only one peak, while CGA or CAF loaded Na-CMC microparticles present a distribution with more than one peak, suggesting there might have been particle aggregation resulting in different groups or clusters of particles.

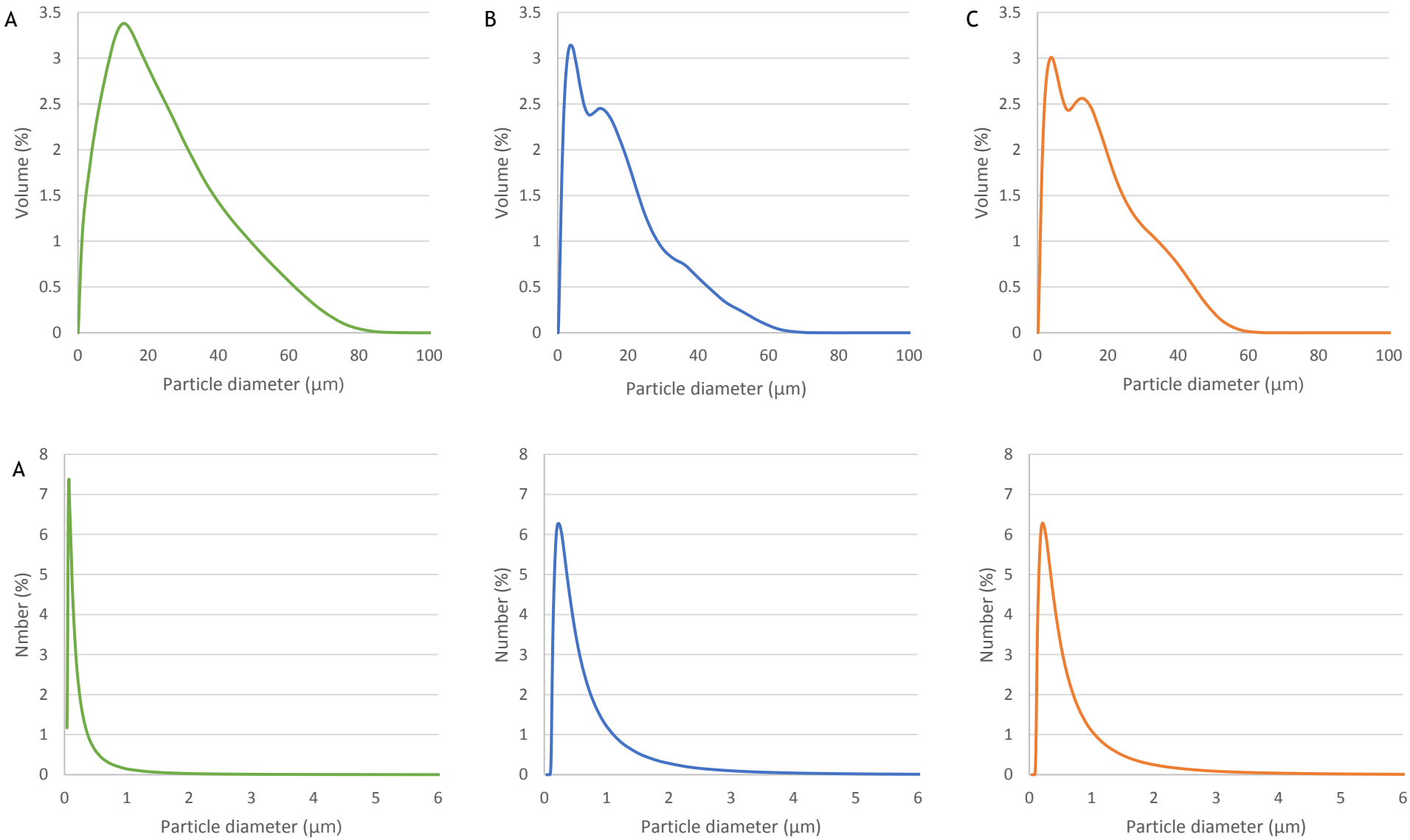


***Figure 12**. Particle size distribution of the microparticles by volume and by number for each sample: A) non-loaded Na-CMC microparticles, B) Na-CMC microparticles loaded with CGA and C) Na-CMC microparticles loaded with CAF.*

5.3.2. Particles morphology

Particles morphology was assessed by SEM analysis. Figure 13 shows SEM micrographs of Na-CMC microparticles loaded with CGA and CAF. It is possible to observe small spherical shape microparticles with smooth surfaces for both CGA and CAF loaded microparticles. Similar results were obtained by other authors. Studies performed by (Sansone et al., 2012) found that Na-CMC spray-dried microparticles containing soybean extracts displayed small and spherical shape microparticles with no pores onto the surface able to promote the loss of the core material. Spherical microparticles, with regular shape were also produced by (Estevinho et al., 2014) after encapsulation of β-galactosidase by spray-drying, using different biopolymers. However, the surface of the microparticles containing β-galactosidase presented different textural characteristics: very rough surface for particles formed with chitosan or arabic gum while particles formed with calcium or sodium alginate or modified chitosan presented a very smooth surface.

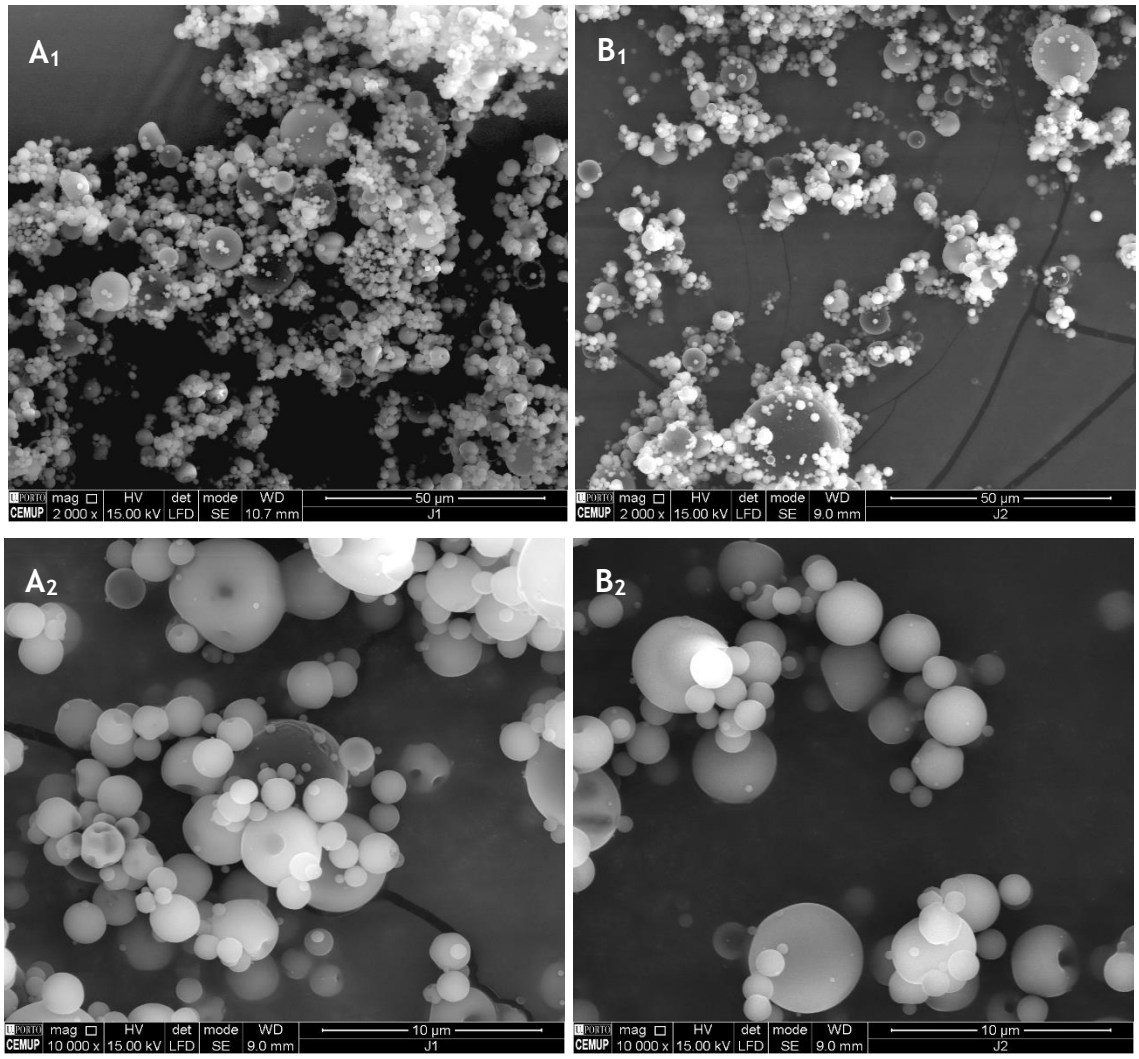


***Figure 13**. SEM micrographs of Na-CMC microparticles loaded with CGA (A) and CAF (B). Amplification of 2000 times is presented in micrographs A1 and B1; amplification of 10000 times is presented in micrographs A2 and B2. (Beam intensity of 15.00 kV).*

5.4. Controlled release studies and entrapment efficiency

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. The release profile of both antioxidants (chlorogenic and caffeic acids) encapsulated with Na-CMC was investigated under aqueous solutions with two different pH values: 5.6 (pH of normal deionized water) and pH 2 (in order to approximate the acidic conditions experienced in the stomach), under magnetic stirring at room temperature (20 – 25 ºC). These preliminary tests were performed by measuring the amount of antioxidant released at different times, for a total period of 24 h. The resulting profiles are displayed in Figure 14, showing the release percentage (amount released at time *t* divided by total released amount), during the time tested.

Overall, results show a similar release for all samples. Firstly, there is a sustained release of the active compound followed by a plateau level on the release profile, which corresponds to maximum release. Being a hydrophilic matrix-forming polymer, Na-CMC is able to swell in the presence of water forming a gel. Particle size also influences controlled release. As previously seen in section 5.3.1., microparticles resulting from the spray-drying process present small particle size and subsequently greater surface area to volume ratio, which favors release.

Despite the resemblances between the release behaviors, there are some differences when considering different medium pH values or core composition.

A decrease on the medium pH value led to a decrease of release time, i.e., a faster release. Microparticles loaded with CGA achieved full release after 2 h at pH 5.6 but only 45 min when release occurred at pH 2. At the same time, microparticles loaded with CAF achieved total release after 45 min at pH 5.6 but only 30 min at pH 2. Therefore, results suggest that release profiles are pH-dependent.

Regarding core composition, total release is achieved faster for caffeic acid than chlorogenic acid, for both pH values tested. Microparticles loaded with chlorogenic acid achieve total release after 2 h at pH 5.6 while microparticles loaded with caffeic acid are completely released after only 45 min. Same behavior seems to happen at pH 2 since CGA microparticles achieve full release later than CAF microparticles (45 min and 30 min, respectively). A possible explanation for these results lies on CAF's smaller size which may have contributed to its faster release.

Similar results were found by (Sansone et al., 2012) while developing a sodium-carboxymethyl cellulose matrix by spray-drying to microencapsulate soy extracts, using different solvent formulations. The resulting microparticles were rapidly soluble in water and were able to release about 80-100% of the bioactive extract in 15-30 min. Other study, performed by (Su et al., 2007), showed different results. Authors encapsulated *R. salvia miltiorrhiza* using a mixture of gelatin and sodium carboxymethyl cellulose and tested their release using a dialysis device containing

pH 2.2 simulated gastric juice which was sealed with a membrane and immersed in the same simulated gastric juice at 37 ºC, 50 rpm. Microparticles achieved only 10% of release of the effective ingredient within the first hour and 75% after about 8 h.

To conclude, results show that Na-CMC microparticles obtained by spray-drying are effective in the sustained release of chlorogenic and caffeic acids. Total release time ranged from 30 min to 2 h, which are acceptable values when comparing to average digestion time. However, further studies are required in order to fully understand how each parameter influences release behavior, namely pH of the release medium and core composition. Full understanding and prediction of release behaviors would be helpful for future food applications of Na-CMC microparticles loaded with coffee antioxidants.

Controlled release studies have also allowed the estimation of the entrapment efficiency. EE was calculated as the ratio between the amount of antioxidant trapped inside the microparticles and the total amount of the antioxidant in the feed solution. The amount of antioxidant trapped inside the microparticles was calculated subtracting the amount of core in water right after dispersion to the total amount of antioxidant in the feed solution. The amount released right after dispersion corresponds to the amount released at time zero, which is considered the amount of core not trapped within the microparticles. As mentioned before, this time zero is relative because it was impossible to measure the absorbance in that precise moment.

Results presented at Table 12 show high values of EE for microparticles encapsulated both with CGA (91%) and CAF (93%), indicating that the present method allows a successful encapsulation of antioxidants within a Na-CMC matrix.

Table 12. Entrapment efficiency results.

Microparticles formulation	Entrapment efficiency (%)
Na-CMC + CGA	91
Na-CMC + CAF	93

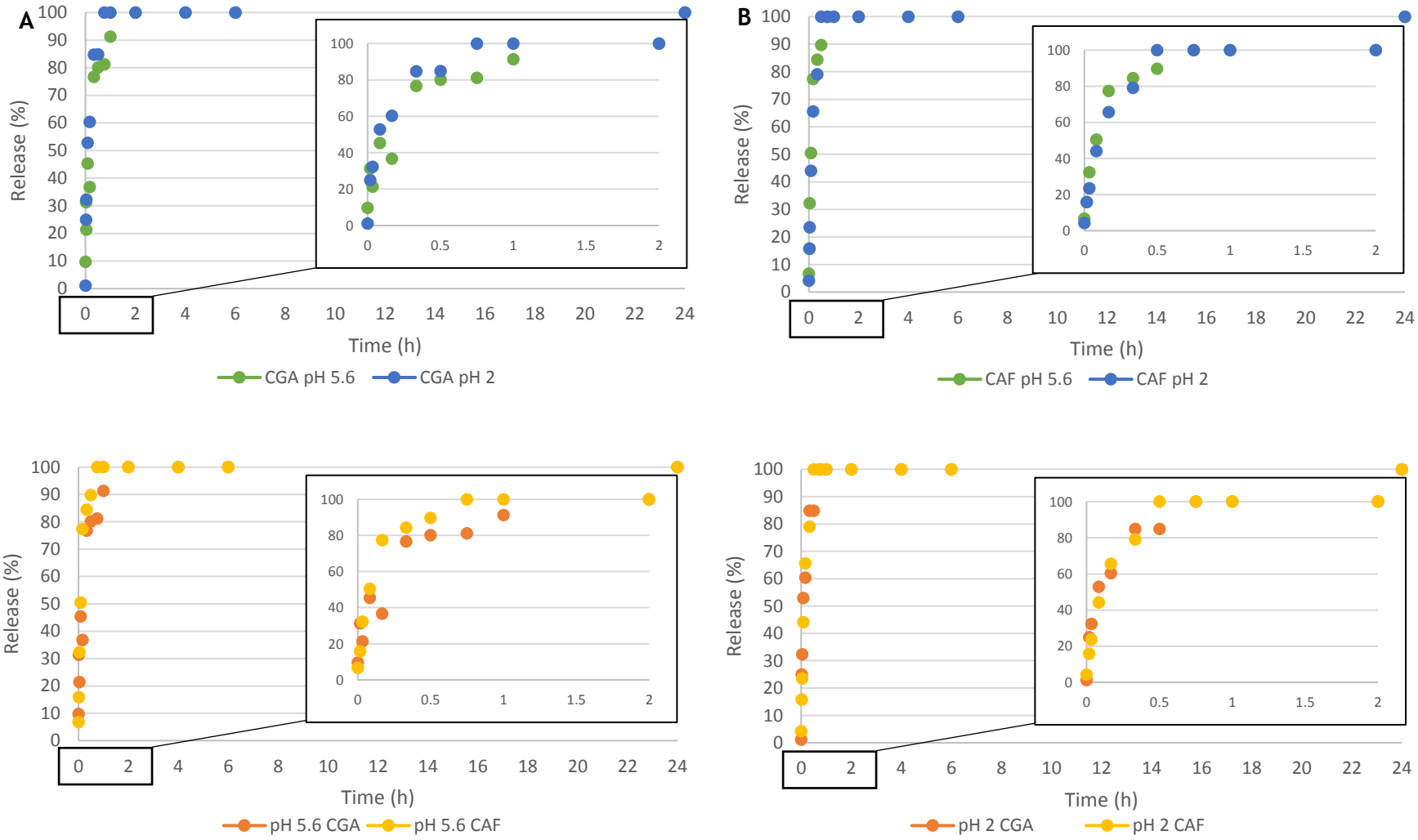


Figure 14. Release profiles of the Na-CMC microparticles: A) Release of Na-CMC microparticles loaded with CGA in pH 5.6 and in pH 2; B) Release of Na-CMC microparticles loaded with CAF in pH 5.6 and in pH 2; C) Release of Na-CMC microparticles loaded with CGA and CAF in pH 5.6 and D) Release of Na-CMC microparticles loaded with CGA and CAF in pH 2.

5.5. Antioxidant activity assessment

Both chlorogenic and caffeic acids are acknowledged coffee antioxidants and their use within microparticles for food products seems to be a promising application. However, it is imperative to assess if the microencapsulation process has prejudicial effects regarding chlorogenic and caffeic acids properties. Therefore, their antioxidant activity was estimated using the ABTS radical scavenging assay. In this test, the amount of the remaining ABTS radical after reaction with the sample was measured by UV-spectrophotometry. Samples consisted of microparticles loaded with caffeic and chlorogenic acid at maximum release (after 4 h in aqueous solution) and also non-loaded microparticles of carboxymethylcellulose. The antioxidant activity of samples containing CGA and CAF in free solution with the same amount as in the microparticles was also assessed. Results of the antioxidant activity of chlorogenic and caffeic acid, both in the free form or encapsulated with Na-CMC, are shown in Table 13 as μM TE (Trolox Equivalent).

Table 13. Antioxidant activity results.

Samples	Antioxidant activity (μM TE)	
	Free in solution	Encapsulated (after 4 h release in water)
CGA	159.4	206.9
CAF	175.6	208.8
Na-CMC	-	28.1

Results show a slight increase of the antioxidant activity for CGA and CAF after the microencapsulation process. The reasons for this increase are unknown, but the presence of Na-CMC might have contributed to the stabilization of the chlorogenic and caffeic acids, leading to higher antioxidant activities. On the other hand, although Na-CMC does not seem to have considerable antioxidant activity, it might have interfered with the spectrophotometry measurements resulting in a deceptive absorbance reading and subsequently deceptive antioxidant activity value. Nevertheless, it is possible to conclude that the present method and conditions are suitable for chlorogenic and caffeic acids microencapsulation without compromising its antioxidant capacity.

CHAPTER 6: CONCLUSIONS

The current project consisted in the microencapsulation by spray-drying of coffee antioxidants, namely chlorogenic and caffeic acids, for future food applications, using carboxymethylcellulose as coating agent.

Validation of the analytical method used for the quantification of CGA and CAF during the controlled release assays showed that both methods were considered precise (repeatability and intermediate precision values below 5%, except for the lowest concentration) and accurate (accuracy values close to 100%). CGA calibration curve was linear from 0.50 to 14 $mg.L^{-1}$ and CAF calibration curve was linear from 0.25 to 15 $mg.L^{-1}$. The limit of detection was 0.10 $mg.L^{-1}$ for CGA and 0.29 $mg.L^{-1}$ for CAF while the limit of quantification was 0.34 $mg.L^{-1}$ for CGA and 0.96 $mg.L^{-1}$ for CAF. The product yield values obtained after the spray-drying process were satisfactory considering the used method and scale. Na-CMC microparticles showed similar yield values: 37.6% for microparticles containing CGA and 40.8% for microparticles containing CAF. Particle size distribution found that microparticles loaded with CGA presented mean diameters of 8.37 μm (considering volume distribution) and 0.44 μm (considering number distribution). For the case of the microparticles loaded with CAF, mean diameters were 8.78 μm (considering volume distribution) and 0.41 μm (considering number distribution). However, non-loaded Na-CMC microparticles showed slightly different mean diameters: 13.49 μm (considering volume distribution) and 0.16 μm (considering number distribution), suggesting that the presence of these antioxidants influenced the mean size of the Na-CMC microparticles. The microstructural analysis confirmed the existence of small spherical shape microparticles with smooth surfaces for both CGA and CAF loaded microparticles. Controlled release experiments showed similar release profiles for all samples. Total release times were: 2 h for CGA at pH 5.6, 45 min for CGA at pH 2, 45 min for CAF at pH 5.6 and 30 min for CAF at pH 2, which are satisfactory considering the average digestion time. Results suggest that release profiles are core composition dependent (faster release for CAF than CGA) and pH dependent (faster release at pH 2 than pH 5.6). Antioxidant activity was higher after the spray-drying process than in free solutions, showing that the chosen methodology can improve the CGA and CAF antioxidant capacity.

The tests performed during this work demonstrate the potential of Na-CMC spray-dried microparticles as carriers of coffee antioxidants, showing great encapsulation efficiency, total release ability and compounds stability without loss of antioxidant activity. However, it should be borne in mind that the present results are only preliminary studies and further experiments should be implemented.

CHAPTER 7: LIMITATIONS AND FUTURE WORK

The main limitations endured during the development of this project included material and equipment availability, such as the spray-dryer, UV-Vis spectrophotometer and magnetic stirrer. Other factor that limited the obtained results was the short time available. Otherwise, it would be possible to repeat the assays and obtain more statistically reliable results. Also, the lack of more suitable equipment might also be considered a drawback. More specifically, in the controlled release studies, the use of a spectrophotometer able to continuously measure the release of one specific sample instead of having to prepare a separate sample for each time measurement would lead to more exact results and a less time-consuming preparation.

For future work, it would be interesting to test different pH values for the release medium in order to better understand the effect of this factor in the release profile. However, the tested pH values should not be higher than 10 in order to avoid antioxidants degradation and because gastrointestinal conditions never reach pH values this high. Also, more complex gastrointestinal simulated conditions could also be experimented for *in vitro* release of microparticles (e. g. medium containing pepsin at pH 1.2 for gastric conditions and phosphate buffer containing pancreatin at pH 7.4 for intestinal conditions). *In vivo* absorption of the antioxidants in the gastrointestinal tract should also be tested to ensure their delivery. Microencapsulation using other cellulose derivatives (e.g. hydroxypropyl or hydroxypropylmethyl cellulose) as encapsulating agents could also be performed in order to verify which type is more adequate for the encapsulation of coffee antioxidants while maintaining the dietary fiber component in the resulting microparticles. One of the main objectives of microencapsulation techniques is the conservation of the bioactive ingredient. Therefore, long-term stability of the microparticles and antioxidants should also be evaluated by measuring the CGA and CAF content after a certain time period (days, weeks and months). An obvious next step is the incorporation of the microparticles into food products. Dried food products such as cookies, bakery products, pasta and doughs would be a proper choice in order to avoid or minimize antioxidants release. Incorporation should be optimized in terms of incorporation yield, long-term stability of antioxidants and their antioxidant activity, controlled release efficiency after ingestion, among others. Also, the manufacturing process of each food product should be considered in order to ensure the microparticles resistance throughout the incorporation. Therefore, studies should be performed in order to evaluate microparticles delivery, stability and overall behavior during food processing (physical, chemical or thermal) and storage. Also, changes in food properties such as texture, moisture, color, quality as well as other sensory properties should also be assessed. Furthermore, extraction of coffee antioxidant from coffee by-products such as spent coffee grounds for microencapsulation purposes would be a favorable experiment from a sustainable, and perhaps economic, point of view.

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APPENDIX

A. Validation of analytical method

The validation of the selected analytical method (UV-vis spectrophotometry) was done in order to ensure its suitability for chlorogenic and caffeic acid quantification and to prove that the method can produce accurate and precise results within the scope of its intended use. A calibration curve was performed by plotting the absorbance values (AU) against each corresponding concentration value ($mg.L^{-1}$). In order to be validated, a calibration curve needs to comply with the following parameters:

- Having at least 5 different standard solutions (with different concentrations);
- Linearity range factor greater than 10;
- $R > 0.995$;
- $\frac{s_a}{a} \times 100 \leq 5\%$;
- $b - ts_b < 0 < b + ts_b$,

where R represents the linearity coefficient, a the sensitivity, b the intercept of the regression and s_a and s_b as the corresponding standard deviations.

Validation performance parameters such as precision (intra-day and inter-day) and accuracy as well as the limits of detection (LOD) and of quantification (LOQ) were also determined. The limit of detection (LOD) is the minimum concentration from which it is possible to detect the presence of the desired compound, with a certain statistical certainty, i.e., with a precision and accuracy that cannot be those obtained for greater concentrations. On the other hand, the limit of quantification (LOQ) is the minimum concentration of the compound which can be measured with the degree of accuracy and precision defined in the method. Equations for their calculations are the following:

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

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

Regarding precision and accuracy, these parameters were assessed using three of the standards concentration of the calibration curve: the highest, lowest and intermediate concentrations. Repeatability (or intra-day precision) is equal to the coefficient of variation (%CV) of six measurements of each sample using the same operator, same solutions, same equipment and same operating conditions, i.e., six measurements on the same day. For intermediate precision (or inter-day precision) estimation, two measurements were performed on three consecutive days. Both precision values should be lower than 5%. Accuracy is measured by the recovery percentage (%R) and is equal to the ratio between the obtained and the expect resulted from the calibration curve.

A.1. Chlorogenic acid

Prior to the calibration curve, an absorption spectrum for CGA in water was performed (Figure A1.1), revealing that the wavelength for maximum absorbance was at 323 nm. Future absorbance measurements were performed at this wavelength.

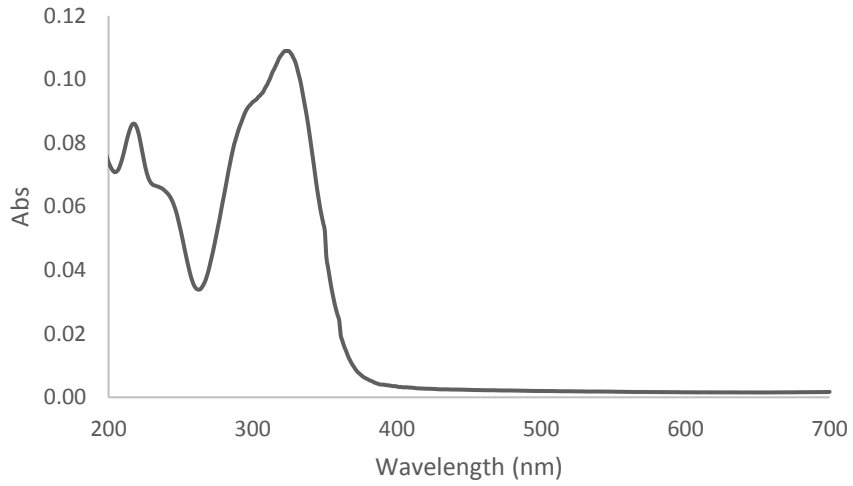

Figure A1. 1. Absorption spectrum for chlorogenic acid in water.

A calibration curve was performed by plotting the absorbance values (AU) against each corresponding concentration value (mg.L^{-1}). Calibration range was 0.5 to 14 mg.L^{-1} (0.5, 1, 2, 4, 6, 8, 10, 12 and 14 mg.L^{-1}) and measurements were done in duplicate. The resulting calibration curve is presented in Figure A1.2.

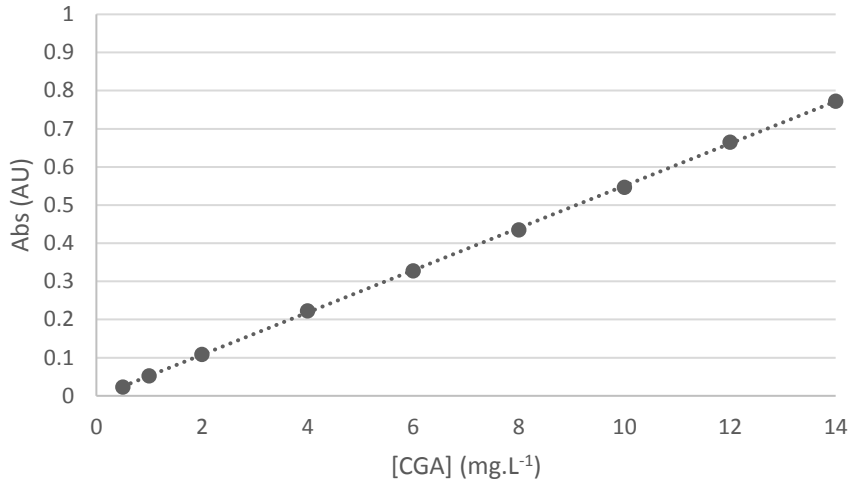

Figure A1. 2. *Calibration curve for chlorogenic acid in water.*

The corresponding linear regression equation was the following:

$$Abs\ (AU) = 0.0554 \pm 0.0002\ [CGA]\ (mg.L^{-1}) - 0.003 \pm 0.002 \qquad \text{(Eq. A1. 1)}$$

In order to be validated, a calibration curve needs to comply with certain parameters. Table A1.1 presents those parameters and corresponding compliance for the obtained calibration curve as well as the LOD and LOQ.

Table A1. 1. *Parameters and restraints required for validation of a calibration curve and corresponding compliance of the obtained calibration curve for chlorogenic acid in water.*

Parameter	Required restraint	Obtained results
Number of standards	≥ 5	9
Linearity range factor	≥ 10	28
Correlation coefficient (R)	> 0.995	1.000
Standard deviation of the slope	$\leq 5\%$	4.3%
LOD ($mg.L^{-1}$)	-	0.10
LOQ ($mg.L^{-1}$)	-	0.34

Validation performance parameters such as precision (intra-day and inter-day) and accuracy were also determined and are presented in Table A1.2.

Table A1. 2. Repeatability, intermediate precision and accuracy obtained for three of the standards concentration of chlorogenic acid in water.

Standards ($mg.L^{-1}$)	Repeatability (Intra-day precision) (%CV)	Intermediate Precision (Inter-day precision) (%CV)	Accuracy (R%)	
1.0	1.4	22.1	77.3	
6.0	0.1	4.5	97.5	91
14.0	0.1	1.3	99.2	

Overall, the present method complies with the required constraints, is precise and accurate, making it suitable for CGA quantification in water.

A.2. Caffeic acid

Prior to the calibration curve, an absorption spectrum for CAF in water was performed (Figure A2.1), revealing that the wavelength for maximum absorbance was at 313 nm. Future absorbance measurements were performed at this wavelength.

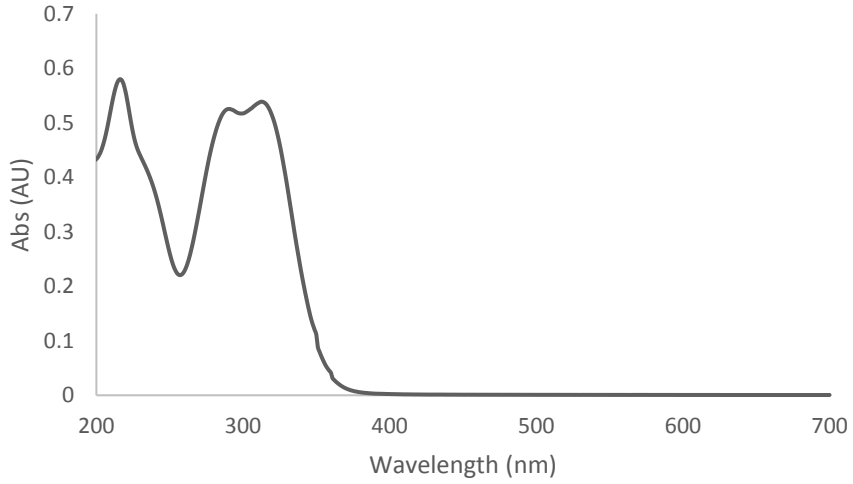

Figure A2. 1. Absorption spectrum for caffeic acid in water.

A calibration curve was performed by plotting the absorbance values (AU) against each corresponding concentration value ($mg.L^{-1}$). Calibration range was 0.25 to 15 $mg.L^{-1}$ (0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12 and 15 $mg.L^{-1}$) and measurements were done in duplicate. The resulting calibration curve is presented in Figure A2.2.

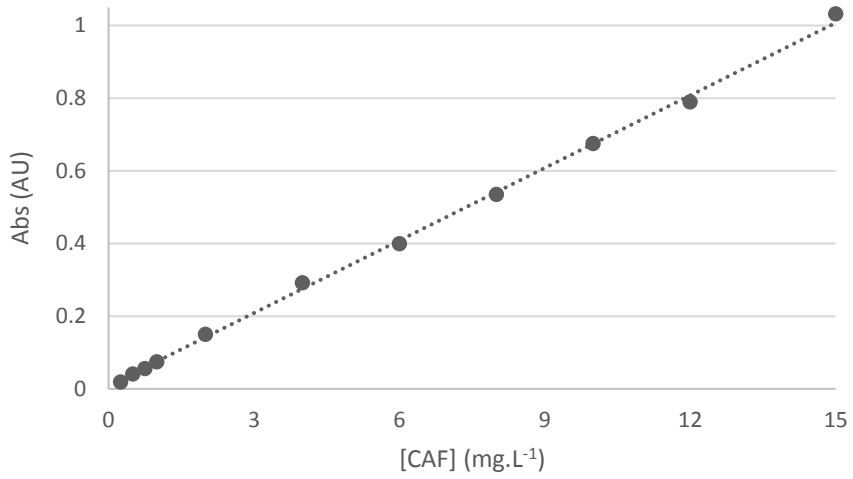

Figure A2. 2*. Calibration curve for caffeic acid in water.*

The corresponding linear regression equation was the following:

$$Abs\ (AU) = 0.067 \pm 0.001\ [CAF]\ (mg.L^{-1}) + 0.009 \pm 0.006 \quad \text{(Eq. A2.1)}$$

In order to be validated, a calibration curve needs to comply with certain parameters. Table A2.1 presents those parameters and corresponding compliance for the obtained calibration curve as well as the LOD and LOQ.

Table A2. 1*. Parameters and restraints required for validation of a calibration curve and corresponding compliance of the obtained calibration curve for caffeic acid in water.*

Parameter	Required restraint	Obtained results
Number of standards	≥ 5	11
Linearity range factor	≥ 10	60
Correlation coefficient (R)	> 0.995	0.999
Standard deviation of the slope	≤ 5%	1.2%
LOD ($mg.L^{-1}$)	-	0.29
LOQ ($mg.L^{-1}$)	-	0.96

Validation performance parameters such as precision (intra-day and inter-day) and accuracy were also determined and are presented in Table A2.2.

Table A2. 2. Repeatability, intermediate precision and accuracy obtained for three of the standards concentration of caffeic acid in water.

Standards ($mg.L^{-1}$)	Repeatability (Intra-day precision) (%CV)	Intermediate Precision (Inter-day precision) (%CV)	Accuracy (R%)	
1.0	0.6	15.7	86.6	
6.0	0.1	3.0	96.5	95
12.0	0.1	1.9	102.1	

Overall, the present method complies with the required constraints, is precise and accurate, making it suitable for CAF quantification in water.

B. Antioxidant activity: Trolox calibration curve

Antioxidant activity was evaluated using the ABTS assay. In this assay, ABTS is previously oxidized by potassium persulfate to generate radical cation $ABTS^{\bullet+}$ (Figure B1). After reaction with the sample, the amount of the remaining ABTS radical was measured by UV-Vis spectrophotometry (at 734 nm).

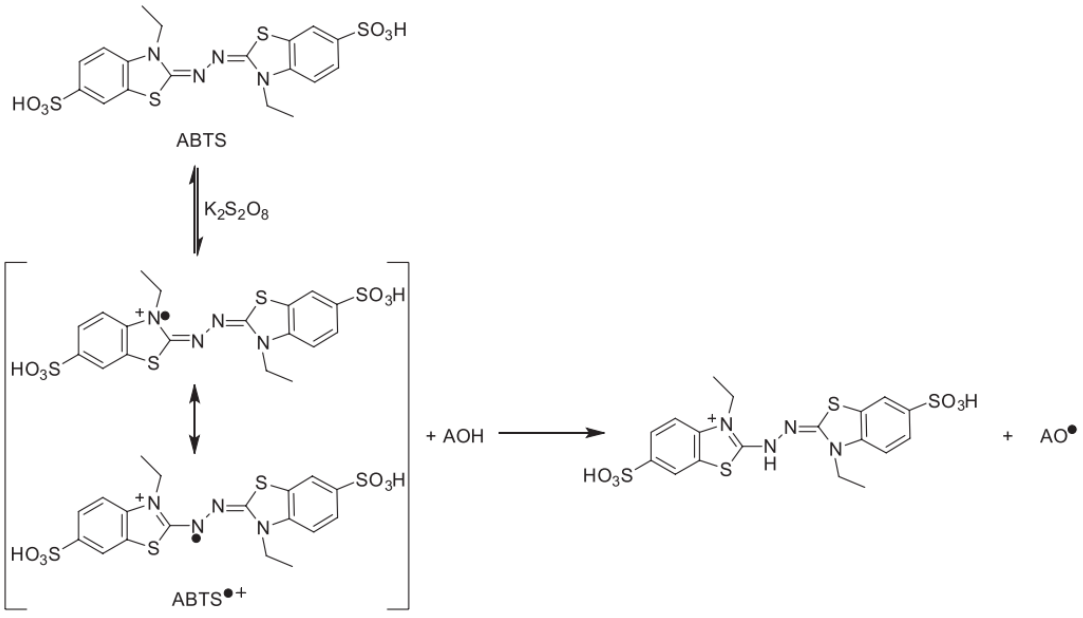

Figure B. 1. Oxidation of ABTS by potassium persulfate to generate radical cation ABTS•+ and its reaction with an antiradical compound (AOH) (Oliveira, Souza, & Eckert, 2014).

Antioxidant activity was expressed in μM Trolox equivalents (TE), a synthetic analogue of vitamin E commonly used as antioxidant reference. The standard curve and equation is presented in Figure B1.

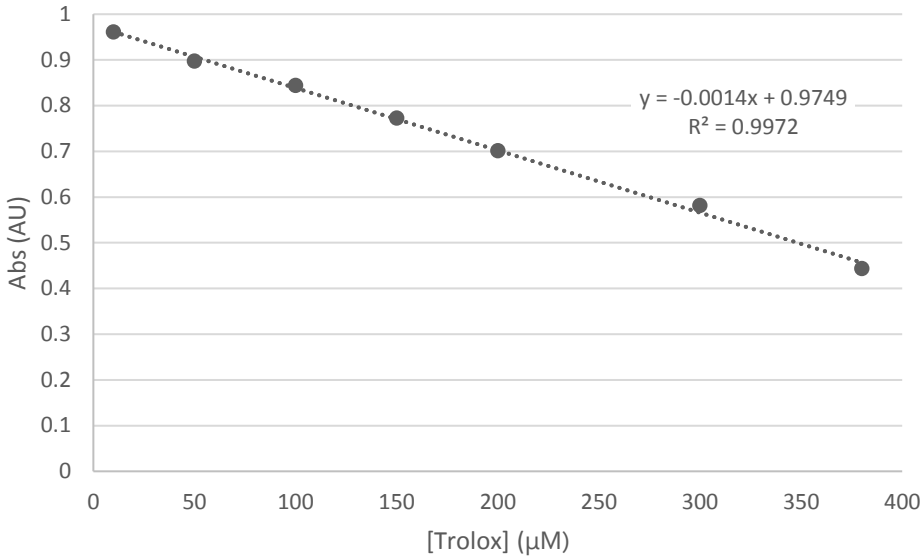

***Figure B. 2**. Linear regression for antioxidant activity estimated by ABTS method.*