Universidade do Minho
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Strategies for valorization of amaranth *(Amaranthus cruentus)* grain nutritional components – behavior under *in vitro* gastrointestinal digestion and in Caco-2 cellular model

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Dissertation for PhD degree in Food Science and Technology and Nutrition

Supervisors of the thesis
António Augusto Martins de Oliveira Soares Vicente, PhD
Joana Teresa Rodrigues Martins, PhD
Ana Cristina Braga Pinheiro, PhD

February 2020

AGRADECIMENTOS

Gostaria de agradecer a todos aqueles que estiveram comigo durante estes 4 anos. Em primeiro lugar, meu Orientador, Professor António Vicente, por todo o apoio, força e carinho. E minhas co-orientadoras incansáveis e super prestativas, Joana Martins e Ana Cristina, que me ensinaram a escrever e tiveram comigo uma paciência imensa. À Universidade do Minho, agradeço a oportunidade de realizar este trabalho. Especialmente, ao Departamento de Engenharia Biológica pelo espaço e equipamentos disponibilizados no decorrer deste trabalho.

A todos os Funcionários, em especial a técnica Dra Paula Pereira, do Departamento de Engenharia Biológica, da Universidade do Minho, pelo apoio e delicadeza em passar informações importantes. Ao Professor José Teixeira pela indicação do meu orientador ainda quando eu estava no Brasil a elaborar o projecto. Aos colegas e amigos do Departamento de Engenharia Biológica, especialmente aos do Laboratório de Instalações Piloto, por tudo que me proporcionaram no decorrer do Doutoramento, aprendizado diário. Agradeço aos meus amigos queridos: Leilson, Priscilla, Carol. Esperanza e Thaís por serem amigos dentro e fora da Universidade. A querida e estimada Carla Faria, minha estagiária, que foi um presente recebido. E a minha querida amiga Catarina, a quem doulei e que se tornou amiga e apoiadora na reta final do meu doutoramento.

Aos colegas e Professores de outras Instituições, que tive o privilégio de conhecer durante a realização deste trabalho, em particular a pesquisadora Idalina Gonçalves da Universidade de Aveiro (CICECO) por toda a ajuda, apoio e estímulo nas experiências lá realizadas e a professora Paula Ferreira pelo auxílio e disponibilização do equipamento SEM, tempo e dedicação.

Por fim, um agradecimento muito especial à minha família que estavam ao meu lado na alegria e na tristeza, na saúde e na doença, e sempre tiveram carinho e amor ao longo destes 4 anos. Aos meus pais e meus antepassados a toda força e energia de conseguir que puderam me dar. Os amo muito. E a minha filha, nascida dentro desses 4 anos, luz da minha vida. E ao João Filipe, meu companheiro, pai exemplar e amor. Ao CNPQ pelo suporte financeiro concedido através da bolsa, a elaboração deste trabalho foi suportada por uma bolsa de doutoramento financiada pelo CNPQ com a referência IF/00300/2015 e financiada pelo Programa Ciência sem Fronteiras. Muito obrigada por tudo!

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Strategies for valorization of Amaranth (*Amaranthus cruentus*) grain nutritional components – behavior under *in vitro* gastrointestinal digestion and in Caco-2 cellular model

Amaranth is a pseudocereal of great nutritional value that has a higher amount of fiber and protein than the cereals usually consumed, which is why it has stood out as an excellent alternative or complementary source of proteins, together with the fact that it does not contain gluten.

In this context, the main objective of this study was to explore the amaranth grain by using innovative technologies, developing and characterizing materials that can be used in food, valuing this grain. In order to achieve this objective, two approaches were developed in this thesis: 1) development and characterization of amaranth protein- and starch-based encapsulation structures and 2) development and characterization of biopolymeric film matrices incorporating amaranth phenolic compounds.

In the first approach, β-carotene was the bioactive compound chosen to be encapsulated in amaranth starch and protein microcapsules formed by spray-drying, aiming at improving its water solubility, stability and bioavailability. Moisture content, morphology, solubility, particle size, encapsulation efficiency and β-carotene stability were analyzed. All microcapsules showed a wide particle size distribution and also low solubility and moisture content. Also, the results showed that encapsulation strongly increased β-carotene stability. Its stability in various *in vitro* food simulants was demonstrated in acidic and neutral media, but not in alcoholic media. Subsequently, a static digestion model was used to assess β-carotene bioaccessibility and its citotoxicity and cellular antioxidant capacity was determined when in contact with Caco-2 cells. The results showed that encapsulated β-carotene presented higher bioaccessibility than free β-carotene. Moreover, β-carotene incorporated in amaranth starch and protein microcapsules was not cytotoxic at 160x dilution. Encapsulated β-carotene presented higher cellular antioxidant capacity than free β-carotene indicating that microcapsules exhibited the ability to protect β-carotene and its antioxidant function.

In the second approach, alginate and glycerol-based films with the incorporation of phenolic compounds extracted from the amaranth grain were developed. The films were produced using different alginate, glycerol and phenolic compounds concentrations and characterized (e.g. mechanical properties, opacity and water vapor permeability). Chemical interactions were studied by FTIR and film surface was analyzed by scanning electron microscopy. Alginate films incorporating amaranth phenolic compounds have been successfully manufactured. The light transmittance of the films was reduced due to phenolic compounds incorporation. Phenolic compounds addition to alginate films also resulted in intermolecular interactions, which was confirmed by FTIR spectra. If mechanical stability is a priority, films presenting higher alginate/glycerol ratio should be produced. On the other hand, if film flexibility is prioritized, the alginate/glycerol ratio must be equal to one.

In general, the two products developed and explored in this thesis can be potential vehicles for functional compounds incorporation. Bioactive compounds entrapment in amaranth polymeric matrices and the use of its functional components provide a new and promising alternative for amaranth grain use and valorization.

Keywords: bioactive compounds, bio-based films, biotechnology, microcapsules, pseudocereal.

RESUMO

Estratégias para valorizar as propriedades nutricionais do grão de amaranto (*Amaranthus cruentus*): comportamento sob digestão gastrointestinal *in vitro* e em células Caco-2

O amaranto é um pseudocereal de grande valor nutricional que apresenta uma quantidade de fibras e proteína superior aos cereais normalmente consumidos, razão pela qual se tem destacado como uma excelente fonte alternativa ou complementar de proteínas e por não conter glúten na sua composição amilácea. Neste contexto, o objetivo principal deste estudo foi explorar o grão de amaranto pela utilização de tecnologias inovadoras, desenvolvendo e caracterizando materiais que possam ser utilizados em alimentos, valorizando este grão. Para atingir este objetivo, duas abordagens foram desenvolvidas nesta tese: 1) desenvolvimento e caracterização de estruturas de encapsulamento à base de proteína e amido de amaranto e 2) estudo, desenvolvimento e caracterização de filmes biopoliméricos incorporando compostos fenólicos extraídos do amaranto.

Na primeira abordagem, o β-caroteno foi o composto bioactivo escolhido para ser encapsulado em micropartículas de amido e proteína de amaranto formadas por pulverização, para melhorar a sua solubilidade em água, estabilidade química e biodisponibilidade. Analisaram-se o conteúdo de humidade, morfologia, solubilidade, tamanho de partícula, eficiência de encapsulação e estabilidade do β-caroteno nas microcápsulas. Observou-se que todas as microcápsulas apresentaram ampla distribuição de tamanho de partícula e também baixa solubilidade e teor de humidade. A estabilidade do β-caroteno encapsulado foi avaliada e os resultados demonstraram que o encapsulamento aumentou fortemente a sua estabilidade. Foi também estudada a sua estabilidade em vários meios alimentares de libertação *in vitro* e verificou-se a sua estabilidade em meios ácidos e neutros, mas não em meios alcoólicos. Posteriormente, foi utilizado um modelo de digestão estática para avaliar a bioacessibilidade do β-caroteno encapsulado e o seu comportamento na presença de células Caco-2, em termos de citotoxicidade e de capacidade antioxidante celular. Os resultados demonstraram que o β-caroteno encapsulado apresentou maior bioacessibilidade que o β-caroteno livre, e que o sistema não é citotóxico a partir de 160 x para as microcápsulas de amido de amaranto e microcápsulas de proteínas de amaranto.. A capacidade antioxidante das microcápsulas mostrou-se elevada a nível celular, indicando que estas exibem a capacidade de proteger a célula e desempenhar a sua função antioxidante.

Na segunda abordagem, foram produzidos e caracterizados filmes à base de alginato e glicerol com a incorporação de compostos fenólicos extraídos do grão de amaranto. Os filmes foram produzidos utilizando diferentes concentrações de alginato, glicerol e compostos fenólicos e caracterizados em termos de propriedades mecânicas, opacidade, teor de humidade, solubilidade e permeabilidade ao vapor de água. As interações químicas foram estudadas por FTIR e a superfície do filme foi analisada por microscopia eletrónica de varrimento. Produziram-se com sucesso filmes de alginato incorporados com fenólicos de amaranto. A transmitância da luz no filme foi diminuída pela incorporação de compostos fenólicos. A adição de compostos fenólicos nos filmes de alginato também resultou em interações intermoleculares, o que pode ser confirmado pelos espectros de FTIR. Se a maior estabilidade mecânica for uma prioridade, devem produzir-se filmes com maior proporção de alginato/glicerol. Por outro lado, se a flexibilidade dos filmes for priorizada, a razão alginato/glicerol deve ser igual a um.

Em geral, os dois produtos desenvolvidos e explorados nesta tese podem ser um potencial veículo para a incorporação de compostos funcionais. Tanto o aprisionamento em matrizes poliméricas do amaranto quanto a utilização dos seus componentes funcionais são uma nova e promissora alternativa para a utilização e valorização do grão de amaranto.

Palavras-chave: compostos bioativos, biofilme, biotecnologia, microcápsulas, pseudocereal.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

a_w – Water activity

EB – Elongation–at–break

L – Thickness

MC – Moisture content

OP – Opacity

RSA – Radical scavenging activity

SD – Standard deviation

T – Temperature

T_g – Glass transition temperature

T_m – Peak melting temperature

TS – Tensile strength

WVP – Water vapour permeability

WVTR – Water vapour transmission rate

YM – Young modulus

Greek symbols

Δp – Water vapour partial pressure difference

ΔE – Total colour difference

ΔH_m – Enthalpy change of melting

Abbreviations

ABAP – 2,2′–Azobis (2–methylpropionamidine)

ABTS – 2,2'–azino–bis(3–ethylbenzothiazoline–6–sulphonate)

ACE – Angiotensin–converting enzyme

AAPH – α′–Azodiisobutyramidine dihydrochloride

ALG – alginate content

AMSBC – Amaranth starch microcapsules with β–carotene

AMPBC – Amaranth protein microcapsules with β–carotene

ANOVA – Analysis of Variance

ASTM – American Society for Testing and Materials

ATR – Attenuated Total Reflectance

BC – β–carotene

CA – Contact angle

CAA – Cellular Antioxidant Activity

CFC – concentration of phenolic compounds

CMC – Carboxymethyl cellulose

DCFH–DA – 2′,7′–Dichlorofluorescin Diacetate

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl sulfoxide anhydrous

DPPH – 2,2–diphenyl–1–picrylhydrazyl radical

DSC – Differential scanning calorimetry

DTG – Derivative thermogravimetry

EO – Essential oil

Gly – glycerol content

FAO – Food and Agriculture Organization of the United Nations

FDA– Food and Drug Administration

FDA/PI – Fluorescein diacetate and propidium iodide

FRAP – Ferric–reducing antioxidant power

FTIR – Fourier transform infrared

GAE – Gallic acid equivalents

GI – Gastrointestinal

HDL – High–density lipoprotein

HPLC – High performance liquid chromatography

HPSA – Amaranth starch hydrolysate

HUM – Moisture conte

IR – Infrared spectroscopy

MTT – 4,5–Dimethyl–2–thiazolyl–2,5–diphenyl–2H–tetrazolium bromide

N.A. – Not applicable

NPs – Nanoparticles

NPU – Net Protein Utilization

ORAC – Oxygen radical absorbance capacity

PBS – Phosphate buffered saline

PC – Principal component

PCA – Principal Component Analysis

PSC – Rapid peroxyl radical scavenging capacity

RAE – Retinol Activity equivalents

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulfate

SEM – Scanning electron microscopy

SGF - Simulated gastric fluid

SIF - Simulated intestinal fluid

SSF - Simulated salivary fluid

TBA – Thiobarbituric acid

TEAC – Trolox Equivalent Antioxidant Capacity

TGA – Thermogravimetric analysis

TGAP – TGA peak

TOSC – Total oxyradical scavenging capacity

TRAP – Total radical–trapping antioxidant parameter

UHPLC – Ultra Performance Liquid Chromatography

USA – United States of America

WPI – Whey protein isolate

SCIENTIFIC OUTPUT

This thesis is based on the following publications:

PAPERS IN JOURNALS WITH PEER REVIEW:

✓ Coelho, L., Silva, P., Martins, J., Pinheiro, A. and Vicente, A., (2018). Emerging opportunities in exploring the nutritional/functional value of amaranth. *Food and Function*, 9, 5499-5512. https://doi.org/10.1039/C8FO01422A.

✓ Coelho, L.M., Gonçalves, I., Ferreira, P., Martins, J.T., Pinheiro, A.C., Vicente, A.A Development and characterizion of β-carotene microcapsules produced with Starch and Protein from amaranth grain. *Journal of Food Science and Technology* (submitted).

✓ Coelho, L.M., Faria, C., Madalena, D., Martins, J.T., Pinheiro, A.C., Genisheva, Z., Vicente, A.A., Development and characterization of alginate films with phenolic compounds obtained from amaranth grain. *Journal of Polymers and the Environment* (submitted).

✓ Coelho, L.M., Pinheiro, A., Martins, J., Vicente, A. Bioaccessibility and toxicity of β-carotene microcapsules produced with starch and protein from amaranth grain. *Innovative Food Science and Emerging Technologies* (submitted).

ABSTRACTS IN PROCEEDINGS OF CONFERENCES

✓ Coelho, L., Pinheiro, A., Martins, J., Vicente, A. Evaluation of nutritional compounds in amaranth grain (Amaranthus *cruentus L.*) for potential food applications. Paper in Conference Proceedings. 13º Semana de Química de Alimentos da Sociedade Portuguesa de Química, Porto, Portugal, September 14-16, 2016.

✓ Coelho, L.M., Gonçalves, I., Ferreira, P., Coimbra, M.A., Martins, J.T., Pinheiro, A.C., Vicente, A.A. Development and characterization of β-carotene microcapsules composed of starch and protein extract from amaranth. 19th Gums and Stabilisers for the Food Industry Conference. Berlin, Germany, June 27-30, 2017.

✓ Coelho, L.M., Martins, J.T., Pinheiro, A.C., Genisheva, Z., Vicente, A.A., Characterization of nutritional compounds in amaranth grain (*Amaranthus cruentus* L.): phenolic compounds extraction and characterization. 2nd International Conference on Food Bioactives and Health 2018. Lisbon, Portugal, September 26-28, 2018.

✓ Coelho, L., Pinheiro, A., Martins, J., Vicente, A. Bioaccessibility and caracterization of β-carotene microcapsules produced with starch and protein from amaranth grain. 8th International Symposium on "Delivery of Functionality in Complex Food Systems". Porto, Portugal, July 07-10, 2019.

SECTION I

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

This chapter introduces the background information, motivation and outline of this thesis, as well as its main objectives.

1.1. RESEARCH BACKGROUND AND MOTIVATION

The global protein demand for the 7.3 billion inhabitants of the world is approximately 202 million tonnes annually (Henchion et al., 2017). Plant-based protein ingredients are becoming more popular due to their contribution to environmental sustainability and to food security challenges, in addition to their cost-effectiveness, compared with animal-based proteins (Aiking, 2011). Quinoa, amaranth, and buckwheat are non-conventional sources of protein that have been the subject of limited studies in recent years, although their cultivation goes back thousands of years (Haros and Schoenlechner, 2017).

In addition, consumers' current quest for lifestyle changes and good eating habits brings the demand for more traditional products with characteristics that coherently defend a favorable nutritional intervention with quality and diversity. Thus, the food industry has made efforts in the development, reformulation or modification of products through their nutritional enrichment with functional ingredients or through technological processes (Day et al., 2009; Mitsuoka, 2014).

Pseudocereals are gluten-free dicotyledon grains which are used and consumed as grains, though botanically pseudocereals are neither grasses nor true cereal grains. Pseudocereals are typically high in protein and other nutrients, gluten free and are considered whole grains. Many so-called "ancient grains" are pseudocereals. These pseudocereals include amaranthus, buckwheat and quinoa (Alonso-Miravalles and O'Mahony, 2018).

Amaranth is a plant originated in Central America, very common in the diet of pre-Columbian America (Amaya-Farfan et al., 2005; Tosi et al., 2001). In recent decades, amaranth cultivation has reappeared not only in Mexico and Central America, but has also spread throughout Latin America, Asia, Europe, and some African countries (Escudero et al., 2004). In the 1980s, the National Academy of Science (USA) ranked it among the 36 most promising cultures to feed humanity. The potential of amaranth as a source of nutrients is high, which has generated interest in some countries to use it as a food source (Bressani et al., 1989; Capriles et al., 2006). The amaranth plant is integrally used: the leaves are consumed as vegetables and the seeds as cereal, and can be used for both human and animal nutrition (Escudero et al., 2011; Martirosyan et al., 2007). Archaeological studies conducted in Peru, Mexico, and the United States reported amaranth cultivation in the region before Christ period. The Incas and Aztecs recognized the importance of

this food and included it in their religious ceremonies, but their consumption was prohibited by the Spanish colonists by the association of this food with the pagan rituals (Bressani et al., 1993; Teutonico and Knorr, 1985).

Amaranth belongs to the class of dicotyledons and family of amaranths. Its species are morphologically similar and have small seeds with lenticular shape, from 1.0 to 1.5 mm and weight from 0.6 to 1.2 mg. The plant can reach 2 m in height. The deep roots of the amaranth plant favor the most efficient use of water, allowing its cultivation in regions of arid and semi-arid climates. Its main agronomic characteristics are: drought, heat and pest resistance; rapid growth; ability to produce large biomass in small space; potential for use as food (Coelho, 2006; Hejazi et al., 2016; Rodas and Bressani, 2009a). More than 60 species of amaranth were identified, three of which were the most consumed and studied: *Amaranthus cruentus* L., *Amaranthus caudatus* L. and *Amaranthus hypocondriacus* L. (Barba de la Rosa et al., 1992; Becker, 1989; Segura-Nieto et al., 1994; Tosi et al., 2001). Due to the characteristics and properties similar to cereal grains, it is considered a pseudocereal (they are not part of the Gramineae family) (Amaya-Farfan et al., 2005; Breene, 1991; Saunders and Becker, 1984; Tosi et al., 2001).

Amaranth grain has nutrients with biochemical activities and physiological potential, such as fibers, squalenes, tocopherols, tocotrienols and phenolic compounds. The easy plant adaptation to different climatic conditions, together with its nutritional and functional proprieties, increases the interest in valuing its use in products with higher added value (Okoth et al., 2017). Although amaranth grain has a well-established chemical characterization and well-studied compounds' physiological potential, few studies have been conducted to evaluate the potential industrial applications of its components.

Over the past decade, new technologies such as biotechnology, genetic engineering and food processing have enabled food scientists to design products best suited to a healthy diet (Correâ-Filho et al., 2019).

In this context, the great amaranth grain potential to improve food properties and to explore new applications of its bioactive or functional compounds in food systems were the main motivations for the development of this thesis.

Figure 1-1 presents the thesis grafical abstract which shows the thesis proposal of using amaranth's components separately, in order to valorize amaranth grain potential use.

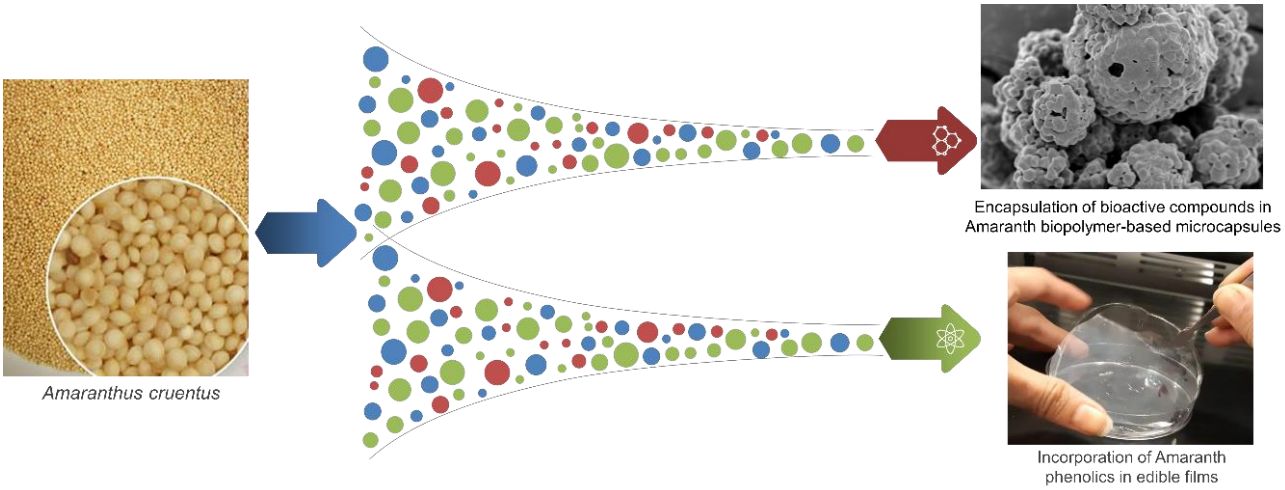

Figure 1-1 - Thesis graphical abstract.

1.2. RESEARCH AIMS

The main objective of this thesis was to develop novel biotechnological vehicles for food application using amaranth grain compounds, in order to explore its inherent functional properties.

The specific objectives of this work were:

- To obtain and chemically characterize the compounds extracted from the amaranth grain;
- To evaluate amaranth grain phenolic compounds' antioxidant capacity;
- To develop and characterize amaranth protein and starch-based encapsulation structures to encapsulate a bioactive compound (i.e. β-carotene);
- To evaluate the effect of an *in vitro* digestion on the bioaccessibility of the bioactive compound encapsulated;
- To evaluate the effect of developed encapsulation structures and encapsulated bioactive compound on viability and antioxidant activity on Caco-2 cells;
- To study the entrapment of amaranth phenolic compounds in other biopolymer matrices (i.e., polysaccharide-based film);
- To evaluate the physicochemical properties (e.g. mechanical and water vapor barrier properties) of alginate-based films after the incorporation of amaranth phenolic compounds.

1.3. THESIS OUTLINE

The results obtained were organized into 4 thematic sections. The first section (Section I – Chapters 1 and 2) provides an overview/bibliographic review on amaranth nutritional and functional components, and the latest amaranth studies and research conducted on biotechnology area. The new data presented in the thesis were organized in two subsequent sections (Section II – Chapters 3 and 4 - and Section III – Chapter 5), corresponding to the potential use of amaranth to produce microcapsules and films. The results reported in Section II correspond to the work conducted with amaranth biopolymers as encapsulating materials for bioactive compounds. Section III presents the results obtained for the characterization of edible films as a vehicle for amaranth phenolic compounds. The last section (Section IV – Chapter 6) contains the most relevant thesis conclusions and future work perspectives.

Section I (Chapters 1 and 2)

This section provides an overview of amaranth grain and its recent food applications. Chapter 1 describes the thesis motivation, the history and the importance of amaranth in the food area, revealing the main research objectives of this thesis. Chapter 2 presents the latest literature research, reviewing the nutritional and functional components of amaranth, and its latest biotechnological applications, from macro to nano-scale, demonstrating its potential.

Section II (Chapters 3 and 4)

Section II describes the microencapsulation of β-carotene using starch and protein extracted from amaranth grain as wall materials, its structure and characterization (Chapter 3). Encapsulation efficiency, particle size, ATR-FTIR, and β-carotene stability were evaluated. In addition, its bioaccessibility after *in vitro* gastrointestinal digestion, toxicity and permeability in Caco-2 cells were evaluated (Chapter 4). The two works complement each other and show that microencapsulation of functional components with amaranth starch or protein can be successfully performed.

Section III (Chapter 5)

Chapter 5 reports the production and characterization of new edible films produced from alginate and glycerol incorporated with amaranth phenolic compounds and the evaluatione of films' composition on their properties. Films physical, transport and mechanical properties are described. The *in vitro* antioxidant capacity of phenolic-containing films has been also tested.

Section IV (Chapter 6)

The final chapter of the thesis highlights the contributions and conclusions derived from the work presented, including a discussion of future perspectives and the work to be developed.

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CHAPTER 2

LITERATURE REVIEW

This chapter gives an overview of the main topics discussed in this thesis. It describes the relevance of amaranth as a pseudo-cereal, its nutritional importance, its recent food applications as coatings/films, emulsifiers and/or as material for encapsulation of bioactive compounds.

The results presented in this Chapter were adapted from:

Coelho, L., Silva, P., Martins, J., Pinheiro, A. and Vicente, A., (2018). Emerging opportunities in exploring the nutritional/functional value of amaranth. *Food and Function*, 9, 5499-5512. https://doi.org/10.1039/C8FO01422A.

2.1. INTRODUCTION

Recently, consumers' knowledge has changed substantially regarding the association between health and diet. The role of diet, in addition to basic nutrition, gained greater recognition. Not only is nutrition aimed at satisfying hunger and providing essential nutrients, it also contributes to the prevention of associated nutritional diseases, reduces health risks and improves human well-being (Barbé et al., 2014; Betoret et al, 2011).

Amaranthus spp. (Amaranthaceae family), commonly known as amaranth, consists of 60 species, which according to the uses for human consumption can be divided into grain and vegetable amaranth (Mlakar et al., 2010). The amaranth plant shows panicle type inflorescences, being frequently classified as pseudocereal, since it not belongs to the grass family and does not contain gluten (such as quinoa). Over the last few years, this has led amaranth grain to become a popular food among people with celiac disease (Thompson, 2001; Aguilar et al., 2015).

Amaranth is a tropical plant that is still not fully explored. Amaranth was an important food crop in the Aztec, Inca, and Mayan societies. Amaranth was a part of the daily diet, in addition to corn and beans. The main cultivation area was in Mexico and around the year 1,400; it is estimated that more than 20,000 tons/year of amaranth was harvested for food purposes (Tosi et al., 2001). However, its production declined notably after the collapse of Central American cultures and returned to interest after the 1990s (Alvarez-Jubete et al., 2010), with many studies carried out at the beginning of the 21st century, occurring a momentary lack of interest from the scientific community between 2008 and 2014. However, amaranth has been raising again the interest of food technologists, which can be seen by the return of publications in recent years that explore more its unknown functional aspects due to the evolution of new characterization techniques (Błaszczak et al., 2015; Montoya-Rodríguez, et al., 2015). More recently, Akinola and Eresama (2010) suggested that China is the world's largest producer of amaranth, both for grain consumption and fodder plant use. Other important amaranth production countries are United States, Canada and Argentina. However, there is no official data on world production levels. Amaranth is one of the few crops in which leaves are consumed as vegetables and grains as cereals. The leaf, known as bledos in Central America, is greatly appreciated as greenery and flowers are used to color clothes (Ferreira, Guerra-Matias, and Arêas, 2007). However, it is the amaranth grains that have actually been used for human food in many ways. The most common

use is to grind them into flour to use in the production of breads, pancakes, cereals, cakes or other flour-based products. The grains can be popped or flocculated as porridge, being a food with cereal characteristics, being gluten-free and more nutritious (Rastogi and Shukla, 2013).

Amaranth grains and their products are a rich resource of bioactive compounds with antioxidant properties (Klimczak, Małecka, and Pachołek, 2002; Piecyk et al., 2009). These non-saponiferous substances of the lipid fraction of grains that include squalene, tocopherols, sterols and others, have antithrombotic, antioxidant, hypocholesterolemic, antidiarrheal, antidepressant and anticancer effects (Breene, 1991; Danz and Lupton, 1992; Gorinstein et al., 2007; Hussain et al., 2009; Kumar et al., 2014; Lehmann, 1996; Lehmann, Putnam, and Qureshi, 1994; Muthukumar and Matheswaran, 2015; Sabbione et al., 2016). Besides, it is important to highlight that amaranth grain protein has high lysine content, and therefore, it constitutes an optimum nutritional supplement for conventional cereals that are poor in this amino acid.

However, the ingestion of large amounts of amaranth bioactive compounds is not directly related to the compounds' bioavailability in blood and tissues due to their low absorption in the digestive tract or rapid metabolic transformation and elimination (Kroon et al., 2004; Manach et al., 2004; Olthof et al., 2003). Only the study of the -amaranth bioactive compounds' bioavailability/bioaccessibility, *in vivo* or at least *in vitro*, will allow to understand the amounts we are obtaining and how we can more easily access these nutritionally relevant components. Besides, it is important to mention that the studied amaranth species reported in this review did not present toxicity effects.

Current research seeks to isolate, use and apply bioactive compounds of amaranth in several food applications as well as to increase bioavailability of those compounds. Figure 2-1 shows the potential new food applications of amaranth grain components recently reported in the literature. These applications are discussed in more detail later in this review.

In this context, this literature review summarizes the present knowledge about amaranth composition and antioxidant capacity, limitations in its intake, strategies to improve its digestibility, bioaccessibility and bioavailability. Furthermore, future prospects about amaranth bioactive compounds' encapsulation in different matrices, especially using nanotechnology methods for encapsulation, are provided.

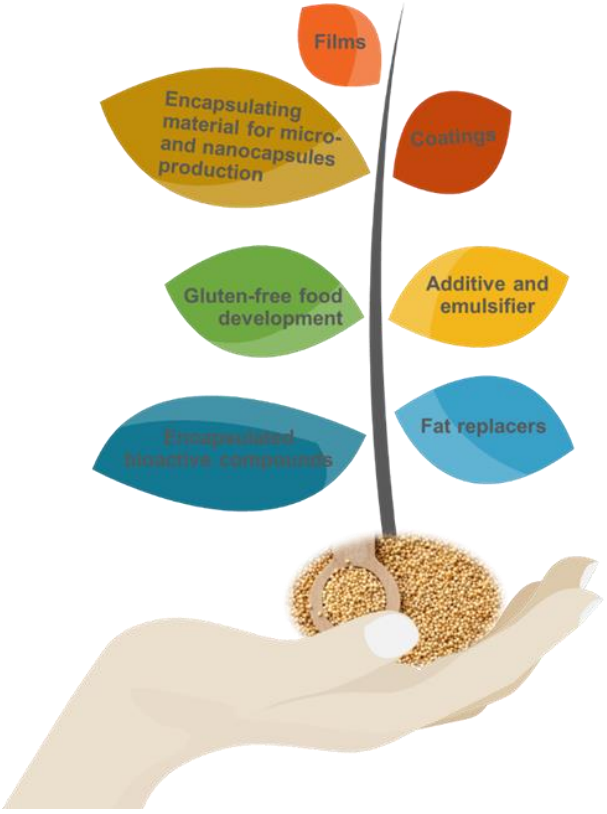

Figure 2-1- Potential applications of amaranth grain components.

2.2 COMPOSITION OF AMARANTH GRAIN

Amaranth grain has been studied for its remarkable agricultural characteristics, such as short growing period and resistance to drought. Amaranth is well known for its high nutritional quality, despite having some anti-nutrient compounds (e.g. phytic acid, oxalates and tannins) that could reduce nutrients' bioavailability, especially its protein content (Najdi Hejazi et al., 2016).

The major species cultivated are *A. cruentus* in Guatemala, *A. hypochondriacus* in Mexico, and *A. caudatus* in Peru and other Andean countries. *A. cruentus* grain, for example, has protein (14.9%), lipid (6.98%) and fiber (4.5%) content higher than other amaranth grain species and higher than other general grains such as wheat (12.3% protein, 8% fat, and 2.3% fiber), corn (8.9% protein, 3.9% fat and 2.0% fiber), rice (7.5% protein, 1.9% fat and 0.9% fiber) and oat (16.1% protein, 6.4% fat, 1.9% fiber) (Saunders and Becker, 1984).

According to several authors (Barba de la Rosa et al., 1992; Breene, 1991; Lehmann, 1996), protein fractions of amaranth are composed of approximately 65% albumin, 17% globulin, 11%

prolamin and 7% of glutelin. According to Martinez and Añón (Martinez and Anon, 1996), the proportion of the different fractions in the isolated protein and its nutritional and functional properties depend on the extraction method used. Albumins and globulins are the main storage proteins in amaranth (Martínez, Castellani, and Añón, 1997). The most important globulins are amaranth 11S, globulin P, and 7S globulin (Avanza and Anon, 2007; Avanza, Puppo, and Añón, 2006; Castellani, Martínez, and Añón, 2000). Albumins are rich in sulfur, amino acids valine and lysine, and globulins are rich in valine, leucine, prolamin, threonine, phenylalanine, leucine, glutelin, histidine and low in lysine (Barba de la Rosa et al., 1992; Segura-Nieto et al., 2005). The 11S fraction is higher than the other fractions due to the presence of various essential amino acids (i.e., tryptophan, methionine, lysine, histidine, phenylalanine, valine and isoleucine) (Marcone, 1999). The content of gliadin, less than 0.01% in some species, renders it useful for the production of food for people with celiac disease (Becker, 1989; Tosi et al., 2001). Moreover, the amount of amino acids leucine is low, 0.54 g/100 g, in amaranth grains, but still larger than rice and corn. The composition shows that 5 % of the amino acids are lysine (reaching up to 6.9%) and 4.4% are sulfur amino acids. These amino acids are limiting in almost all grains (Teutonico and Knorr, 2003). Therefore, amaranth is rated above other grains in terms of nutritional potential due to their content of lysine and other essential amino acids (Breene, 1991; Bressani, Elias, and Garcia-Soto, 1989).

Starch is the main component of the amaranth grain ranging from 48% to 69%, depending on the species (Hoover et al., 2010). Starch granules are polygonal, usually measuring 1-3 μm (Singh and Singh, 2003). Branched amylopectin is the major component of amaranth starch; its molecular weight may vary 1 to 70 x 10^7 Da, in A. hybridus and A. hypochondriacus, as demonstrated by various light scattering techniques (Kong et al., 2008; Kong et al., 2010). Starch granule consists of about 1700 amylopectin molecules (Wilhelm et al., 2002). It was stated that the amylopectin external chains' form double helices responsible for the creation of the crystalline region in the granules, while the inner chains contribute to the development of amorphous regions (Bertoft, 2017; Pérez and Bertoft, 2010).

Amaranth grain is also an excellent source of insoluble fiber, principally lignin and cellulose. According to Becker and Saunders (1984), the total fiber content found in amaranth is higher than that of widespread cereal. Amaranth grain is softer and thinner compared to wheat bran, and presents 16 and 9 % of bran and dietary fiber, respectively (Tosi et al., 2001).

Amaranth grains are a significant source of minerals, for example, *A. cruentus* BRS Alegria,

display contents (per 100 g dry basis) of phosphorus (441 mg), potassium carbonate (434 mg), magnesium (254 mg), calcium (206 mg), sodium (0.6 mg), iron (12 mg), zinc (5.2 mg), manganese (4 mg), aluminum (4 mg), cobalt (0.06 mg) and selenium (0.02 mg). Also, the following vitamin content (100 g on dry basis) are reported for this grain variety: riboflavin (B2) (< 0.03 mg), pyridoxine (B6) (0.05 mg), niacin (8.04 mg), thiamine (0.10 mg), total tocopherol (4.3 mg) and vitamin E (2.1 mg) (Venskutonis and Kraujalis, 2013).

The amaranth oil can also be considered a significant source of linoleic and linolenic acids, previous results indicated that linoleic and stearic acids constitute about 70 and 20 %, respectively, of amaranth lipids (Schoenlechner, Jurackova, and Berghofer, 2005; Yanez et al., 1994). Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is a non-saponifiable lipid which acts as a biosynthetic precursor of all steroids from plants and animals. Several studies suggested that amaranth has hypocholesterolemic, antioxidant and anticarcinogenic effects, due to its favorable profile of unsaponifiable and high fatty acids content, being considered a functional food (Berger et al., 2003; Fritz et al., 2011; Lina, Wakako, and Akihiko, 2006; Plate and Arêas, 2002). Amaranth oil has higher squalene quantities (2.4 to 8.0 %) than other common vegetable oils such as olive oil (0.45 to 0.54%), wheat germ and rice bran (0.1 to 0.7%) (Becker, 1989; He and Corke, 2003). The authors Rodas and Bressani (2009) demonstrated that amaranth oil can be considered an alternative plant source in obtaining squalene, having reported 8 to 12 g/100g amaranth. This percentage varied in proportion to the amount of unsaponifiables isolated during the extraction.

Tocotrienols are unsaturated vitamin E analogues that have shown a regulatory role in cholesterol metabolism, antitumor activity and high *in vitro* antioxidant capacity. Tocopherols and tocotrienols have important antioxidant properties (Escudero et al., 2004; Lehmann et al., 1994). According to Lehmann, Putnam and Qureshi (1994), tocopherols found in several species of amaranth are α-tocopherol (2.97 to 15.65 mg/kg of grain), α-tocotrienol (5.92 to 11.47 mg/kg grain) and β-tocotrienol (0.95 to 8.69 mg/kg grain).

The processing of amaranth can influence the content of micronutrients. For example, steam cooking of *A. hybridus* resulted in 29.2% loss of vitamins. However, the flavonoid content increased by 25%, probably due to flavonoids' release during the cooking process (Adefegha and Oboh, 2011).

A. caudatus and *A. paniculatus* free phenolic acids were isolated using ethanol and purified (Klimczak et al., 2002). It was reported that *A. caudatus* grains presented 16.8 to 59.7 mg/100 g

of phenolic acids (Repo-Carrasco-Valencia et al., 2009). The rutin content of the amaranth leaves and grains was 24.5 and 0.08 g/kg, respectively. Quercetin or its derivatives was only present on mature amaranth leaves (Kalinova and Dadakova, 2009). Seven phenolic acids were quantified in *A. cruentus*; their total quantities were 327.93 ± 18.87 mg/kg in expanded grains and 398.36 ± 22.63 mg/kg in flakes. Vanillic, p-hydroxybenzoic and ferulic acid were the major phenolic acids (> 80 mg/kg each), while caffeic acid constituted less than 5 mg/kg and traces of synapic and cinnamic acids were found in grains (Ogrodowska et al., 2012).

Amaranth grain has low content of antinutritional factors, such as oxalates, nitrates and phenolic compounds, and different studies indicated that these are thermolabile compounds (Chávez-Jáuregui, Silva, and Areas, 2000; Gamel et al., 2005). Thus, it is recommended that amaranth grain is properly processed before consumption. The tannin content is 0.52-0.61% and the phytate levels (inositol hexaphosphate) are 0.043-0.116% in raw amaranth (Lorenz and Wright, 1984). Phytates form insoluble complexes with a diversity of nutrients and thereby, hinder the absorption of proteins and minerals in particular iron, zinc and calcium. The antinutritional effect of polyphenols is the complex formation (with iron and other minerals) and the precipitation of proteins, which reduces their absorption (Michaelsen et al., 2009).

Therefore, amaranth grain products emerge as highly desirable for consumption as food or as enrichment food ingredients of the diet of many communities. The high nutritional value of the protein makes the amaranth grain applicable for the fortification of wheat flour, corn and tubers, as well as the preparation of gluten-free farinaceous products (Aguilar et al., 2015; Breene, 1991; Silva-Sanhcez et al., 2008). Also, its phenolic compounds may be the key in preventing some chronic non-communicable diseases.

2.3 GRAIN AMARANTH AS FOOD

The amaranth grain can be used as whole grain, such as whole-wheat flour, or combined with other cereals. Wheat flour blended with high amaranth protein content can be used to enhance the nutritional worth of the final food product such as noodles, cookies, potatoes, cassava or maize breads and cakes. Amaranth can be used to substitute wheat and other grain products at levels up to 15%, thereby altering significantly the technological functional properties of the products

(Dieterich et al., 1997). Singhal and Kulkarni (2018) reported that amaranth grain starch maize could be used as a thickener in sauces. Various food processing technologies (e.g., germination and lactic acid fermentation) have been proposed as ways to improve nutrient density and reduce antinutrients (Hotz and Gibson, 2007).

Yanez et al. (1994) observed that the native starch of amaranth is resistant to freezing and thawing, and therefore, stable to some types of thermal treatment, although acidic conditions may limit its stability. Candy and snacks made from this grain are nutritious and widely consumed in Asia and South America (Singhal and Kulkarni, 2018).

Amaranth snacks with good acceptance and high nutritional value have been developed by extrusion (Chavez et al., 2004). Besides its high acceptability, amaranth snack also presented hypocholesterolemic effect in rabbits (Plate and Arêas, 2002), high biological value proteins, and high bioavailability (Ferreira et al., 2007).

Amaranth flour is frequently used in mixtures with maize or wheat to obtain a balanced source of proteins (Alvarez-Jubete et al., 2010; Escudero et al., 2004). Also, it has been reported that the nutritional value of bread could be enhance with the addition of expanded amaranth grains (10–20%) as an alternative to amaranth flour (Marija et al., 2008), increasing iron, phosphorus, calcium, magnesium and potassium contents (Pasko et al., 2007; Shukla et al., 2006). Miranda-Ramos, Sanz-Ponce, and Haros (2019) developed bread with high nutritional and technological quality using *Amaranthus spinosus* and *Amaranthus hypochondriacus* whole flour, significantly increasing myo-inositol phosphate, proteins, lipids, fibers, ashes and phosphate contents.

2.4 ANTIOXIDANT CAPACITY OF AMARANTH GRAIN

In recent years, the interest in finding natural antioxidants has grown, because reactive oxygen species (ROS) or free radicals (i.e., hydroxyl radical, superoxide anion and singlet oxygen) react with biomolecules, such as proteins and lipids, causing severe damage to the cell membrane and DNA. There is evidence that these processes are strongly associated with carcinogenesis and degenerative processes (Madhujith and Fereidoon, 2006; Yashin et al., 2017) such as cardiovascular diseases, osteoporosis and others. Therefore, antioxidants play a potential role in the oxidation process by reacting with these free radicals (Piskounova et al., 2015). The compounds

capable of disrupting auto-oxidation can act in two ways: the first one involves the transfer of hydrogen atoms, the free radical captures the hydrogen atom of the antioxidant, resulting in the formation of a stable radical antioxidant and the oxidizing reaction is interrupted; the second is based on electron transfer (Mastorci et al., 2017; Ou et al., 2002). The antioxidant capacity of phenolic compounds is mainly due to their reducing properties and chemical structure. These characteristics play a central role in reducing or scavenging free radicals (such as singlet oxygen) and chelating transition metals by acting on both the initiation and the propagation steps of the oxidative process (Bauhinia et al., 2011; Domazetovic et al., 2017; Naczk and Shahidi, 2006).

Phenolic compounds are mainly located in the outer layers of the grains, acting as protectors against pathogens and pests. For this reason, the content of phenols presented in flour depends on the degree of extraction. The main phenolics in cereals are phenolic compounds and tannins, and they have smaller quantities of flavonoids. These compounds are partially linked to the benefits of a diet rich in cereals (Liyana Pathirana and Fereidoon, 2005; Michalska, Ceglińska, and Zieliński, 2007).

Data from the literature on food polyphenols content and composition are incomplete and scarce to establish the optimal dietary intake. Some studies provided information on isolated ingestion of certain types of polyphenols, such as flavonols (Crozier et al., 2000), flavanones (Manach et al., 2003), catechins (Higdon and Frei, 2003), phenolic acids (Scalbert and Williamson, 2000) and flavon-3-ols (de Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo, 2000), but there is a lack of comprehensive information on the optimal consumption of total polyphenols. Furthermore, the data of polyphenols in foods generally correspond to the analyzed food extracts (i.e., removable polyphenols), and not to bioavailable polyphenols (i.e., absorbed in the intestine) (Guilherme et al., 2012; Liyana-Pathirana and Fereidoon, 2005; Saura-Calixto, Serrano, and Goñi, 2007; Shahidi, 2009). The current estimated consumption of polyphenols is about 100 mg/day (Lee and Aedin, 2006).

The quantity and quality of polyphenols present in amaranth grain may vary due to several factors, such as genetics, soil composition, growing conditions, maturation degree and post-harvest conditions (Faller and Fialho, 2009). For example, polyphenols and tannins profiles and amounts are affected by processing due to its highly reactive nature (Dlamini et al., 2009). Polyphenols have antioxidant potential, besides the potential to bind charged proteins, amino acids and/or polyvalent cations or minerals like iron, zinc and calcium in food (Gilani, Cockell, and Sepehr, 2005). Further

studies are needed regarding compounds' bioavailability, changes during processing and isolation as well as storage and stability.

Amaranth grain contains phenolic compounds (e.g., flavonoids) and relatively high antioxidant capacity (Gorinstein et al., 2007). Barba de la Rosa et al. (2009) identified and quantified three polyphenols (rutin, isoquercitin and nicotiflorin) and three flavonoids (4-hydroxybenzoic acid, syringic acid and vanillic acid) from *A. hypochondriacus*. Rutin and its metabolites can effectively be associated with the prevention of numerous diseases such as neuropathies, joint stiffness, senile cataract, Alzheimer's disease and cardiovascular diseases (Cervantes-Laurean et al., 2006). Nicotiflorin has a protective effect on the decrease of memory impairment (Huang et al., 2007) and recent findings have revealed its role as therapeutic potential in brain ischemia (Li et al., 2006). Rutin was present at higher concentration (10.1 mg/g amaranth flour), while nicotiflorin was present at 7.2 mg/g flour and isoquercitin was present at 0.5 mg/g amaranth flour. Syringic acid (associated with the bitter taste of amaranth), vanillic acid and hydroxybenzoic acid were present at 0.8, 1.8 and 2.0 mg/g amaranth flour, respectively. Repo-Carrasco-Valencia et al. reported very similar values (Repo-Carrasco-Valencia et al., 2010).

The majority of the polyphenols are hydrolysed by enzymes of the digestive tract and/or by the intestinal microflora (Scalbert and Williamson, 2000; Spencer, 2003). The bioavailability of phenolic molecules increases when a higher amount of these compounds break to form monomers and aglycones (Tarko et al., 2009). The aglycones are mostly absorbed in the small intestine as esters, glucosides and polymers, but in their native form, they not pass through the gastrointestinal tract walls (Rios et al., 2002; Saura-Calixto et al., 2007). Glycosylation has a significant influence on polyphenol absorption rate. The characteristics of some flavonoid aglycones skeleton allow their transport through membranes by passive diffusion (Aherne and O'Brien, 2002). Usually, polyphenols are absorbed in small intestine and colon and they are carried to the liver, leading to a variety of conjugated forms (Pandey and Rizvi, 2009). Czerwinski et al. (2004) found that oat and amaranth flour showed antioxidant activity using *in vitro* models. Oats showed higher antioxidant activity (34.6%) than amaranth flour (23.2-26.0%), probably due to the larger amounts of phenolic compounds present in the former. The authors also found that amaranth flour and grains have high antioxidant activity and positively affected the lipid profile in rats fed with high-cholesterol diets. Tsaliki et al. (1999) found that amaranth flour had higher antioxidant activity than soy flour due to the high concentration of total phenolics and phospholipids in amaranth flour. Klimcsak et al.

(2002) observed that two species of amaranth, *A. caudatus* and *A. paniculatus* presented antioxidant activity index between 84 and 88 % using β-carotene-linoleic acid system. Although the two species have shown similar results, their composition, especially their content in phenolic compounds was different.

Proteins present in amaranth are also known for their antioxidant capacity, especially those containing sulfur (cysteine and methionine), aromatic (tyrosine and tryptophan) and lysine, histidine, proline, glycine, alanine and threonine amino-acids (Soares-Freitas et al., 2015). According to Tong et al. (2000), proteins are able to exert this activity by sequestering transition metals and/or free radicals of tyrosine and cysteine amino acids. Delgado et al. (2015) identified some antioxidant peptides (e.g. fraction 11S) produced during gastrointestinal digestion of amaranth proteins. The detected antioxidant peptides exhibited some structural characteristics, all showing no less than one aromatic residue. The most active peptides belong to the internal structure or to exposed regions of the subunit 11S of the globulin structure. In another study, Delgado et al. (2016a) evaluated amaranth peptides generated under *in vitro* gastrointestinal digestion or alcalase hydrolysis, and their ability to scavenge reactive species usually present in the human body. The most active hydrolysate was obtained by direct digestion of the protein isolated from amaranth, as a pre-hydrolysis with alcalase failed to improve the antioxidant ability. The formation of peptides resulted in a strong scavenging activity against peroxyl and hydroxyl radicals and peroxynitrites. These amaranth peptides could potentially restrain initiation or propagation of radical reactions.

Soares et al. (2015) demonstrated that GG, VIVG and VGVL peptides of *A. cruentus* exhibited *in vitro* 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibition. This result suggested that these peptides have a hypocholesterolemic effect. Suárez et al. (2020) analyzed the hypotensive effect of amaranth protein/peptides on hypertensive rats, where all samples produced a decrease in blood pressure. Ayala-Niño et al. (2019) has separated and identified bioactive peptides found in amaranth seeds by enzymatic hydrolysis, where two fractions showed high biological activity.

Therefore, the importance of analyzing amino acids and polyphenols isolated from amaranth, and studying their bioavailability and bioaccessibility is fully justified, together with their stability and efficacy in human health.

2.5 AMARANTH PROCESSING AND NUTRITIONAL CHANGES

Food processing can induce significant changes in the chemical composition and amount of bioactive compounds of plant origin (Miglio et al., 2008). Processing may modify the polyphenol content of foods in various ways (Manach et al., 2004). Studies on different products after cooking have shown that the total polyphenols content and antioxidant capacity is lower compared to fresh foods (Ismail, Marjan, and Foong, 2004; Sultana, Anwar, and Iqbal, 2008; Zhang and Hamauzu, 2004) and higher in some foods, such as green vegetables (Turkmen, Sari, and Velioglu, 2005).

Traditionally, the amaranth grain is placed in a hot frying pan for toasting (Solanki et al., 2018). In this case, there is little or no control over the temperature, as well as the residence time of the seeds exposed to high temperatures. However, information/studies related to the effect of amaranth processing on the content of bioactive compounds (e.g. phenolic compounds) and antioxidant capacity is still rather scarce. Shevkani et al. (2014) pointed out the effect of lipids and proteins in the amaranth flour physicochemical and functional properties' and found a great change by removal of lipid/degreasing, improving flour and starch stability during heating. Also Muyonga, Andabati and Ssepuuya (2014) studied the effect of various processing methods on amaranth grain (*A. hypochondriacus* and *A. cruentus*) physicochemical properties' namely antioxidant activity and protein digestibility. These authors observed that *A. cruentus* exhibited higher protein content and antioxidant activity than *A. hypochondriacus*. Moreover, they found that protein digestibility and antioxidant activity were affected when the two amaranth varieties were exposed to dry heat. Popped grain had a higher negative effect on protein digestibility, whereas roasting reduced antioxidant activity. Heat processing also leads to changes in the viscosity and rheological behavior of amaranth. Queiroz et al. (2009) showed that all amaranth grain processing reduced the total phenolic content. However, amaranth grain extrusion, popcorn and roasting processes did not reduce the inhibitory oxidation activity when compared to the raw grain in the β-carotene-linoleic acid assay. Perales-Sánchez et al. (2014) demonstrated that the best parameters' combination to maintain (and even improve) amaranth nutritional values (i.e. highest antioxidant activity, total phenolic content and flavonoid values) was the production of germinated flour at 30 °C during 78 h of germination.

Germination increases free amino acids, carbohydrates, fibers and other components levels

and also increases the grains functionality due to the successive increase of the bioactive compounds (Frias et al., 2002). Studies have reported that the germination processes caused a significant increase in the polyphenol content and antioxidant capacity of lupine seeds (Frias et al., 2005), pea and sprouted soybean meal (López-Amorós, Hernández, and Estrella, 2006) and saracen wheat (Kim, Kim, and Park, 2004). Alvarez-Jubete et al. (2010) demonstrated that germination increased the total phenol content and antioxidant capacity of wheat, quinoa and amaranth grains when compared to that found in raw or roasted grains.

The extrusion process uses high temperature and pressure which lead to starch gelatinization and protein denaturation (Chávez-Jáuregui et al., 2003). Montoya-Rodríguez et al. (2015) demonstrated the role of hydrolysis time on the amaranth peptide profile, where lower size amaranth hydrolysates were obtained when hydrolysis time was extended. The extrusion process had a greater impact on the peptide profile, producing more peptides with biological activity. Also, it was shown that unprocessed and extruded amaranth flours are sources of peptides with biological activity (e.g. ACE inhibitor peptides) related to the prevention of major chronic diseases.

Amaranth grain processing under conditions that do not damage proteins and their amino acids bioavailability, such as cooking with moist heat and extrusion, resulted in a final product with good protein quality (Bressani, de Martell, and de Godinez, 1993). However, it is essential to mention that, depending on the method applied before amaranth consumption, processing could affect total phenolic compounds and antioxidant activity of the final product.

2.6 STRATEGIES TO IMPROVE DIGESTIBILITY, BIOACCESSIBILITY AND BIOAVAILABILITY OF AMARANTH GRAIN

Throughout the years, nutrients' digestibility, bioaccessibility and bioavailability have been studied by several *in vitro* screening methods, which can give helpful information about the factors that can influence nutrient absorption. Bioavailability, which is defined as "the amount of an ingested nutrient that is absorbed and available for physiological functions, is dependent on digestion, release from the food matrix, absorption by intestinal cells, and transport to body cells". In turn, bioaccessibility is the "amount of an ingested nutrient that is potentially available for absorption, being thus dependent only on digestion and release from the food matrix" (Carbonell-Capella et al., 2014; Etcheverry, Grusak, and Fleige, 2012; Ringling and Rychlik, 2017).

It was reported that net protein utilization (NPU) (defined as the fraction of nitrogen intake that is absorbed) of several amaranth species range between 33.56 and 46.04%, similar to maize (51.1%) and wheat (40.3%) (Aguilar et al., 2015; Arellano et al., 1996; Lúquez de Mucciarelli et al., 1990). *In vitro* digestibility of amaranth protein is 75.40%, which was considered to be a good digestibility compared to other proteins of the same origin (Aguilar et al., 2015; Escudero et al., 2004; Mendoza and Bressani, 1987). Najdi Hejazi et al. (2016) showed that the nutritional quality and digestibility of amaranth grain could be improved by low-cost malting and amaranth grain germination pretreatment. For instance, germination at 28 °C for 48 h resulted in better protein digestibility (84%). Furthermore, the content of phytic acid and oxalate decreased during the process, while tannin increased. Capriles et al. (2008) studied the amaranth starch *in vitro* digestion and showed that amaranth is a food with a high glycemic index. Starch content was quickly digested, and the hydrolysis and glycemic indexes have been significantly increased by processes of grain roasting, roasting and grinding. This could indicate that processed amaranth grains are digested more rapidly and starch is better digested than raw grains, cooked and extruded. Cooked, stewed and extruded amaranth grains showed starch digestibility similar to that of white bread (reference sample), while flaked and toasted grain had a slightly higher capacity to increase glycemic response. Several characteristics of the amaranth grain can explain the high starch digestibility. These include small size of starch granules, reduced contents of resistant starch and soluble fibers, high levels of amylopectin and low temperature of gelatinization which, when achieved during processing, lead to a loss of the crystalline and granular structure of the amaranth starch (Capriles et al., 2008).

Another study evaluated the scavenging capacity of digested amaranth protein isolates by simulated gastrointestinal digestion. The results showed that isolates obtained by gastrointestinal digestion increased the scavenging capacity against peroxyl radicals, hydroxyl radicals and peroxynitrite (Delgado et al., 2016b). Sabbione et al. (2016) evaluated the potential antithrombotic activity of amaranth protein isolate submitted to simulated gastrointestinal digestion. A fraction of the protein amaranth obtained by enzymatic treatment showed higher antithrombotic activity than the original amaranth protein. Additionally, the *in vitro* absorption through the intestinal epithelium (i.e., Caco-2-TC7 cells) of this active protein fraction was studied and it was observed that some peptides are able to cross the cell monolayer.

The intestinal absorption of nutrients and bioactives (e.g., phenolic compounds) occurs in

different quantities and depends on the food matrix. When they reach the bloodstream, the metabolites of these compounds bind to plasma proteins (e.g., albumin), that transport these substances (Halliwell, Rafter, and Jenner, 2005). The absorption rate depends also on the nature of sugar present in the molecule. When a sugar molecule is attached forming a glycoside derivative, flavonoids' hydrophilicity increases, thereby reducing the possibility of passive diffusion (Day et al., 2001). The active β-glucosidases present in the intestinal epithelium (responsible for dividing the β-glycosidic bonds from active aglycones) are lactase-phlorizin hydrolase and β-glucosidase cytosolic. The first is located in the small intestine wall in the outer surface of the epithelial cells, while the latter is found in liver, kidney and small intestine (Pandey and Rizvi, 2009). Moreover, phenolic compounds can also get to the colon as conjugates or derivatives and glycosides are converted in phenolic acids by gut microflora (Manach et al., 2004; Pandey and Rizvi, 2009). Different conjugated forms could be produced in the liver through the metabolism of the products of polyphenolic compounds' digestion absorbed in the small intestine and colon. Flavonoids are partially bioavailable in humans, although portal blood facilitates removal of the absorbed material to maintain a favorable concentration gradient (Wittig et al., 2001). Absorption and bioavailability of bioactive compounds (usually present in amaranth) has been shown to be higher for isoquercetin, lower for quercetin and lower for rutin as observed in humans (Olthof et al., 2003). In fact, in humans, around 93 % of quercetin is metabolized in the gut. In addition, aglycone is not detectable in human plasma and body tissues in relevant amounts, although its conjugates, gluconate, sulfate and methylated quercetin are documented in humans and animals (Lesser, Cermak, and Wolffram, 2004). Also, quercetin bioavailability was shown to be 17% as compared to 100% in intravenous quercetin, as reported in pigs (Ader, Wessmann, and Wolffram, 2000). In rats, rutin was monitored to be absorbed slower than quercetin, as rutin is probably hydrolyzed by microflora, whereas quercetin was absorbed directly from the small intestine and large intestine (Manach et al., 1997).

One of the few studies that mention strategies to increase bioavailability and bioaccessibility of amaranth nutrients demonstrated that amaranth extrusion increased its calcium bioavailability, assessed by rats' tibia and femur weights and calcium and phosphorous content of rats' bones (Ferreira and Arêas, 2010). However, it is not clear yet how the extrusion mechanism can increase calcium absorption and bone incorporation. Extrusion cooking could produce a chain of chemical transformations on some calcium complexing compounds (e.g. tannins, fiber, phytates and

oxalates) which could lead to the calcium bioavailability enhancement. These results show that this process positively modifies amaranth grains which can be a complementary source of dietary calcium (Ferreira and Arêas, 2010). An iron-amaranth supplementation study conducted in 1990 showed a considerable increase in final hemoglobin, being the hemoglobin gain shown to be due to a more absorbable form of iron (Whittaker and Ologunde, 1990). β-carotene-rich vegetables such as carrots and amaranth were also found to be iron and zinc bioaccessibility enhancers. In another study, carrot and amaranth grains were used (2.5 g and 5 g per 10 g grains, respectively) and these have shown to significantly increase the bioaccessibility of iron and zinc in food grains, being the percentages of this increase between 13.8-86.2% in the case of carrots and between 11-193% in the case of amaranth (Gautam, Platel, and Srinivasan, 2010). Subramanian and Gupta (Subramanian and Gupta, 2016) studied bioavailability and pharmacokinetic of NO_3 and NO_2 from amaranth extract (2 g single dose) in 16 healthy subjects in a cross-over design during 24 h. Results showed that amaranth extract significantly increased NO_3 plasma and saliva levels when compared to placebo group. Moreover, NO_2 plasma level was higher ($p<0.05$) in the amaranth test group compared to the placebo group. These results suggested that the increase of NO_3 and NO_2 levels may help to reduce aging-related disorders and increase high-performance in sport (Subramanian and Gupta, 2016).

To date, no published studies have directly compared the amaranth components (e.g., phenolic compounds) relative bioavailability, although there are some studies available on these individual components isolated from other sources (Angelino et al., 2017; Carbonell-Capella et al., 2014; Chitindingu, Benhura, and Muchuweti, 2015). As an essential step forward regarding the study of the bioaccessibility of food components, dynamic GI models have been developed. For example, TIM-1™ has been used to measure phenolic compounds' bioaccessibility. Moreover, colonic fermentation experiments can be incorporated into this model thus, the polyphenols bioaccessibility assessment may be more trustworthy. This model, which mimics the GI tract (through the duodenum, jejunum and ileum) was used to examine the anthocyanin bioaccessibility in blackberry and wild blueberry (Lila et al., 2012). These authors observed that between the second and third hour, after ingestion, the majority of the anthocyanins were bioaccessible.

All examples above showed a common issue: the attempt to enhance the food nutritional value. Nevertheless, it is essential to overcome many limitations, since just a small part of bioactive ingredients remains bioavailable after oral ingestion due to 1) insufficient residence time in gastric

conditions, 2) low solubility and/or permeability in the intestine, 3) low stability during food processing and storage (e.g., temperature, oxygen and light), or digestion (e.g. pH and enzymes), 4) interference of food substances with the bioactive compounds, which limits their activity and potential health benefits, and 5) instability problems such as those provoked by autoxidation reactions, epimerization and low availability (Munin and Edwards-lévy, 2011).

In the attempt to overcome the limitations above, encapsulation is another strategy that can be used to increase bioaccessibility and bioavailability of bioactive compounds. This technique consists in the entrapment of a compound by a surrounding material to produce capsules. This structure sometimes can be an efficient barrier against the environment factors such as oxygen, light and free radicals. In addition, encapsulation has the capacity to improve compound controlled release, to protect it throughout the processing, storage and transport (de Vos et al., 2010), and to separate it from other compounds (Souza Simões et al., 2017). Numerous encapsulation techniques (e.g., spray drying, fluidized bed coating, coacervation, liposome entrapment, extrusion and inclusion complexation) have been applied in the recent years to change liquid components into solid particles and to offer a means for bioactive compounds' controlled release (Mandžuka and Knez, 2008). For example, microencapsulation using different biopolymers through spray drying is a subsequent step to be coupled with a suitable extraction technique. Thus, products with improved physicochemical properties (i.e., porosity, stability, solubility and dispersibility) are obtain compared to the original material, allowing easier handling, storage and transportation (Lajovic, et al., 2015). While (micro)encapsulation shows advantages regarding technological and handling features of the (micro)encapsulated food ingredients, in particular phenolic compounds (which are particularly difficult to handle otherwise, due to their intrinsic chemical instability), it should also show advantages in terms of improved bioaccessibility and bioavailability. This can only be done analyzing the gastrointestinal fate of those ingredients, including digestion and also absorption by the intestinal epithelium (desirably *in vivo*, but at least *in vitro*).

The next section is devoted to present a number of studies which isolated amaranth bioactive compounds to 1) improve their functionality and bioavailability and 2) produce new functional amaranth-based structures (e.g., films and capsules).

2.7 POTENTIAL USE OF AMARANTH IN FOOD APPLICATIONS

2.7.1. Amaranth as a raw material for the production of edible films and coatings

Several studies have focused on film and coating development from natural polymers such as amaranth proteins and polysaccharides, and also lipids (alone or in combination) (Bertan, et al., 2005; Ferreira, Alves, and Coelhoso, 2016). Moreover, many research works have focused on optimization of films' production to improve their mechanical (e.g., resistance to breakage and adhesion) and gas barrier properties (Otoni et al., 2017). Sobral (2005) developed *A. caudatus* flour films, and concluded that this material produced films with good gas barrier properties compared to other bio-based films. Although amaranth flour films showed to be highly flexible, they showed relatively low mechanical strength. According to these authors, amaranth films have a complex structure, since they are not only formed by starch (i.e., amylose and amylopectin) and plasticizer, but also possess significant protein and lipid content in their composition. Moreover, native lipids and water also acted as plasticizers, which produced an increase in the elasticity of the films and a reduction in its stiffness.

In another work, Tapia-Blácido et al. (2011), studied and compared the properties of amaranth flour films of two species (*A. cruentus* and *A. caudatus*). It has been shown for *A. caudatus* flour films that lipids were associated with proteins and were homogeneously distributed within the starch network. This characteristic provided improved water vapor barrier properties and better elongation compared to *A. cruentus* flour film. Moreover, these authors demonstrated that the barrier properties of the different films were positively influenced by biopolymers' interactions. The interaction between lipids and protein molecules in the amaranth flour film formed hydrophobic areas that prevent the diffusion of water molecules and consequently, reduced water vapor permeability of the films. In the case of oxygen permeability, it was established that the decrease of this property is also due to the strong interaction between biopolymer chains within the film, leaving a minor free volume, hindering the diffusion of oxygen molecules. Interactions between biopolymers in their natural state in amaranth flour contributed to the mechanical and gas barrier properties of the produced films.

Edible amaranth films were also produced with essential oils from cinnamon, oregano or

lemon grass, and their antifungal activity was evaluated against *Aspergillus niger* and *Penicillium digitatum*. The amaranth films presented antifungal activity; however, it was weaker than chitosan films (Avila-Sosa et al., 2012).

Condés et al. (2018) used starch granules and nanocrystals in amaranth protein films. They obtained homogeneous films, but only starch nanocrystals addition to films caused a significant reinforcing effect in the protein matrix, improving film mechanical and barrier properties.

As previously mentioned, several studies demonstrated the applicability of amaranth protein as material for edible film production (Condés, Añón, and Mauri, 2015; Condés et al., 2015; Diéguez et al., 2015). However, the research associated to the inclusion of bioactive compounds from amaranth in different film matrices could be also considered an efficient alternative for the food industry, since amaranth biological activities could enhance edible films' properties. It is important to highlight that the antioxidant activity of the film incorporating amaranth bioactive additives, such as polyphenolic compounds, depends on compound type and concentration present in the amaranth extracts. Therefore, bioactive compounds compatibility with the biopolymer, stability during processing and storage conditions of the natural compounds should be evaluated. Also, controlled delivery studies of amaranth bioactive compounds, or antioxidant activity of films should be performed.

2.7.2 Amaranth as an additive and emulsifier

Amaranth foams and emulsions prepared with protein hydrolysates have potential as a nutraceutical food in the prevention of chronic degenerative diseases. Furthermore, the antioxidant peptides may also be useful to prevent the generation of ROS, extending the shelf life of food products. Soriano-Santos and Escalona-Buenda (2015) demonstrated that the amaranth surfactant' properties were improved by partial hydrolysis, and also demonstrated angiotensin-converting enzyme activity. In another study, the substitution of 15% of bovine protein in an emulsion-type meat product with amaranth protein concentrate, isolated from five plant genotypes, greatly affected the properties of the cooked meat emulsion and gel. However, some positive effects were obtained only in the K112 genotype (Bejosano and Corke, 1998).

Also, it has been reported that starch from amaranth has potential use as food thickener and fat replacer (Lehmann et al., 1994).

Tikekar et al. (2008) extracted and stabilized squalane from amaranth grains, where squalene showed good stability to be used as a bioactive compound in food or as an emulsifying agent. Moreover, Zhou et al. (2012) added red amaranth pigments in the manufacture of pork sausage at concentrations ranging from 0.1 to 0.3% for NO_2 replacement. The pigments addition increased significantly the color value, taste, and acceptance but significantly reduced thiobarbituric acid (TBA) and volatile basic nitrogen values. Based mainly on the results of the general acceptance during 29 days of storage, it was concluded that amaranth' pigments may be a potential alternative to NO_2.

2.7.3 Amaranth as microencapsulation material for hydrophilic and lipophilic compounds and as source of bioactive compounds to be encapsulated

Microencapsulation can be defined as a technology that produces solid, liquid or gaseous capsules (with a diameter ranging from a few microns to 1 mm) that may release their content at controlled rates and under specific conditions. This technique is based on the development of a polymeric capsule that can control interactions between its internal and external parts (Farheen, et al., 2017). Microcapsules may be used to transport numerous compounds, such as vitamins, minerals, phytosterols, fatty acids, antioxidants and probiotics (Cerqueira et al., 2014; Champagne and Fustier, 2007; de Vos et al., 2010). Since bioactive compounds present in food are subject to rapid inactivation or degradation, they can benefit from microencapsulation that avoids or slows down the degradation process until product consumption (Aditya, Espinosa, and Norton, 2017; Zhang et al., 2015). Recently, considerable interest has been directed to microencapsulation of nutraceuticals in the prevention of diseases.

One of the main factors that influences the functional properties of the microcapsules and hence, the stability of encapsulated compounds, is the nature of the encapsulating material (Cerqueira, Vicente and Pastrana, 2018). Encapsulating materials must meet the following requirements: 1) good film-forming properties; 2) low hygroscopicity; 3) low viscosity at high solids concentrations; 4) mild flavor and odor; 5) easy reconstitution; 6) low cost; 7) semipermeable nature, and 8) spherical morphology, enveloped by a tough solid or solid/liquid membrane (Ramos et al., 2017).

Furthermore, the choice of materials should be based on non-reactivity of the coating material

with the compound to be encapsulated (Martins et al., 2016). In this context, carbohydrates are frequently used as encapsulating materials, for their capability to physically bind to the bioactive compounds, as well as their diversity and lower cost. Proteins and lipids may be also used as materials for encapsulating bioactive compounds (Aditya et al., 2017).

Different studies have shown that biopolymers extracted from amaranth can be used as encapsulating materials. For example, small starch granules from *A. paniculatus* in combination with polysaccharide bonding agents (i.e., gum arabic, carboxymethyl cellulose and carrageenan) were used for the entrapment of a flavoring compound, vanillin. It was shown that the gum arabic with 1.0% amaranth starch gave better encapsulation of vanillin than carboxymethylcellulose and carrageenan. Amaranth starch was a better encapsulation material than the starch extracted from quinoa, rice and colocasia, which was explained by the quantity of amylose in the various starches (i.e., negative correlation of amylose on the degree of entrapment of vanillin) (Tarie et al., 2003). Table 2-1 shows more published studies that demonstrated that it is possible to isolate biopolymers from amaranth and use them as encapsulating materials for bioactive compounds entrapment.

Results demonstrating the possibility of amaranth bioactive compounds' encapsulation in different matrices, especially in food products, are still scarce and should be further exploited. The scientific literature reported few methods related to the encapsulation of bioactive compounds extracted from amaranth, being electrospinning (Aceituno-Medina et al., 2015) the most reported one. The encapsulation of bioactive compounds from amaranth using nanotechnology could be considered one approach to enhance compounds' bioavailability and protection during their passage through the gastrointestinal tract and consequently, to deliver the compound at a particular point, thus taking full advantage of its beneficial effect. Table 2-2 shows published studies that have explored the isolation and encapsulation of bioactive compounds from amaranth.

Table 2-1- Selected examples of amaranth as encapsulating material/immobilization matrix for bioactive compounds entrapment.

Encapsulating Material	Encapsulated Compound	Encapsulation Method	Morphology	Application	Findings	Reference
Amaranth protein isolate; Pullulan	Folic Acid	Electrospinning	Fibre NPs	Photoprotection of folic acid	Increased thermal stability of folic acid	(Aceituno-Medina, Mendoza, Lagaron, and López-Rubio, 2015)
Amaranth	Mexican oregano EO	Film casting	Edible films	Production of antifungal edible films	Antifungal activity of Mexican oregano EO	(Raúl Avila-Sosa et al., 2010)
Amaranth	Theophylline	Gelation	Gel/Paste matrices	Incorporation and release of theophylline in starch matrices	Influence of amylose and amylopectin matrices in theophylline dissolution	(Błaszczak, Buciński, and Górecki, 2015)
Modified amaranth starches	*Bifidobacterium breve*; *Lactobacillus casei*	Spray drying	Microcapsules	Encapsulating material for probiotics for food applications	Excellent viability for storage at 4°C for both encapsulated probiotics	(Cortés et al., 2014)
Amaranth derivatized starches	*Bacillus thuringiensis*	Spray drying	Microparticles	Potential to be used as a bio insecticide formulation	Amaranth formulation showed a high level of insecticidal activity	(Rodríguez, Martínez, Barrera-Cortés, Ibarra, and Bustos, 2015)
Amaranth protein isolate; Pullulan	Nisin A; Pediocin pa-1	Electrospinning	Nanofibers	Potential to be employed in food, textile and biomedical industries	Thermostable electrospun nanofibers with antimicrobial activity	(Soto Martínez, 2014)
Amaranth; gum Arabic; Sodium CMC; Carrageenan	Vanillin	Spray drying	Microspherical aggregates	Flavours entrapment for food application	Better entrapment for amaranth aggregates than other starches	(Tari, Annapure, Singhal, and Kulkarni, 2003)
Amaranth starch; Glycerol; Sodium hypochlorite	-	Film casting	Edible films	Encapsulating agent in edible films to replace gum Arabic	Amaranth starch shows a better film forming ability than corn starch	(Chattopadhyay, Singhal, and Kulkarni, 1997)

Table 2-1 Continued

Encapsulating Material	Encapsulated Compound	Encapsulation Method	Morphology	Application	Findings	Reference
Oxidized amaranth starch; Glycerol; Sodium hypochlorite	Vanillin	Film casting	Edible films	Oxidised starch as a potential substitute for gum arabic in encapsulated flavour	Reduced hygroscopicity in amaranth starch films	(Chattopadhyaya, Singhal, and Kulkarni, 1998)
HPSA	Orange oil; Lemon oil	Spray drying; emulsion	Microcapsules; emulsions	Encapsulation of sensitive food ingredients	HPSA excellent encapsulating agent for lemon oil but not suitable for orange oil	(Kshirsagar and Singhal, 2008)

HPSA: amaranth starch hydrolysate; CMC: carboxymethyl cellulose; EO: essential oil

Table 2-2- Selected examples of amaranth as bioactive material to be encapsulated/immobilized.

Amaranth Compound	Encapsulating Material	Encapsulation Method	Morphology	Application	Findings	Reference
Protein isolate	Acetic Acid; Sodium Hydroxide	Electrospinning	Fibre NPs	Encapsulating structure for food components	Influence of solvent and protein concentration	(Aceituno-Medina, Lopez-Rubio, Mendoza, and Lagaron, 2013)
Protein isolate	N.A	Gelation	Gel	Application in gel-like food products	Rheological properties at different concentrations and thermal conditions	(M. V. Avanza, Puppo, and Añón, 2005)
Protein isolate	N.A	Gelation	Gel	Ingredient in gel food formulation	Structural properties at different concentrations and thermal conditions	(Maria V. Avanza, Puppo, and Añón, 2006)
Betacyanin extract	Maltodextrins; starches	Spray drying	Powder	Betacyanin as a food grade colorant.	Betacyanin extracts have increased storage stability and reduced hygroscopicity	(Cai and Corke, 2000)
Oil	WPI;Sodium caseinate; Maltodextrins	Spray drying; emulsion	Microcapsules; emulsions	Solid powder to be used in pharmaceutical or food products	Better oxidation stability with emulsions; amaranth oil dispersed in microcapsule wall	(González Montes, 2012)
Amaranth (reducing agent and a stabilizer for NPs synthesis)	Iron Oxide; Ferric chloride	NPs synthesis by precipitation	NPs	Environmental, optoelectronics and biomedical applications	NPs with amaranth extract showed better photocatalytic and antioxidant capacity than other NPs	(Muthukumar and Matheswaran, 2015)

NPs: nanoparticles; WPI: whey protein isolate; N.A.: not applicable

2.8 CONCLUDING REMARKS

The present review showed that amaranth is a rich source of food compounds with great importance to improve food products' functionality and to formulate healthier foods.

The encapsulation of amaranth bioactive compounds appears to be an efficient way of introducing these compounds into food products, offering better stability, as well as providing access to their health benefits to a wider range of the population. It was also shown that amaranth biopolymers have great potential to being used as encapsulating agents, once they can act as protective coatings against adverse environmental conditions such as light, moisture and oxygen. However, further work is needed to elucidate the actual contribution of enriched functional foods to improve the welfare of consumers. For this reason, further studies on bioaccessibility and bioavailability, *in vitro* and in vivo, of different formulations containing amaranth and/or amaranth bioactive compounds used in existing or new products are required. In addition, the role of such

bioactive compounds with controlled release systems in the colon should be studied in more detail.

The next innovative steps should focus on identification of antioxidants and phenolic compounds present in amaranth grain extracts and evaluate their potential applications. Moreover, there are no studies on the use of nanotechnology to encapsulate amaranth components or using amaranth biopolymers as encapsulating materials; our research group began to explore this subject. In fact, nanotechnology has been pointed out as one of the most interesting topics in food science. The use of this technology requires studying the behavior of nanosystems as carriers of functional compounds, as well as their controlled release and protection during food processing and oral intake. In this context, it is necessary, to evaluate the full impact of digestion on the bioactivity and bioavailability of amaranth bioactive compounds, their fate in the gastrointestinal tract, and to assess their safety for human consumption.

To achieve the ultimate goal of exploring health benefits from amaranth, in vivo tests will be needed to assess if strategies that demonstrate efficacy in increasing amaranth bioactive compounds bioavailability translate into positive human health outcomes. In fact, studies on the impact of amaranth phenolic acids on the intestinal microbiota and their mechanisms of action in humans are still scarce. The generation of bioavailability data from in vivo studies is time-consuming and expensive. Therefore, *in vitro* reproduction may be a helpful step to investigate factors that influence bioavailability.

As one of the basic principles of functional foods is the functionality of bioactive compounds through multiple metabolic pathways, possibly the favorable amaranth health effects are due to the combined presence of compounds as found in whole grain. Future research should be directed to epidemiological studies and to the consolidation of mechanisms of action, especially in the human body. However, it is also desirable to conduct research to determine the minimum amount of amaranth bioactive compounds that should be consumed through the diet in order to produce the expected effects. Thus, there are still many opportunities to explore the great nutritional and functional potential of amaranth in this field that can be established (Sorrentino et al., 2007; Cushen et al., 2012). Potential risks of nanotechnologies (e.g. nanoparticles) should be assessed on a case-by-case basis until more safety information is known about these new technologies (Silvestre et al., 2011).

This literature review is intended to provide a background perspective of the progress of the scientific community, and introduce the central issues that will be further explored in the

experimental work reported in this thesis.

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SECTION II

AMARANTH AS AN ENCAPSULATING MATERIAL FOR A BIOACTIVE COMPOUND

CHAPTER 3

DEVELOPMENT AND CHARACTERIZION OF β-CAROTENE MICROCAPSULES PRODUCED WITH STARCH AND PROTEIN FROM AMARANTH GRAIN

Starch and protein extracted from amaranth grain were used as matrices for β-carotene microencapsulation by spray-drying. The encapsulation efficiency, microcapsules moisture content, morphology, solubility, particle size, and β-carotene stability were analyzed.

The results presented in this Chapter were adapted from:

Coelho, L.M., Gonçalves, I., Ferreira, P., Martins, J.T., Pinheiro, A.C., Vicente, A.A. Development and characterizion of β-carotene microcapsules produced with Starch and Protein from Amaranth grain. *Journal of Food Science and Technology* (submitted).

3.1 INTRODUCTION

Carotenoids are yellow-orange-red dyes of great interest for food industry that can be naturally found in fruits and vegetables, some fungi, and algae. They also help strengthen the immune system and support the proper functioning of the reproductive system (Galaffu, Bortlik, and Michel, 2015; Sagis, 2015). β-carotene is a carotenoid that presents a wide spectrum of biological activities (e.g., anti-cancer, anti-hypertensive, and anti-inflammatory) due to its free radicals scavenging properties and is therefore considered a very attractive dietary supplement (Cerezo et al., 2012; Lim et al., 2016). However, due to its extremely high hydrophobicity, it is difficult to incorporate β-carotene in food formulations and its bioavailability is fairly low (Gomes et al., 2017; Soukouli et al., 2016). Moreover, this carotenoid also needs to be protected from exposure to heat and light because any changes in β-carotene chemical structure such as cyclization, double bond migration, and oxygen molecule addition, definitely results in loss of its biological action.

These facts lead us to investigate an efficient system for the enrichment of the antioxidant activity in a food formulation through β-carotene addition, without the mentioned losses (Eriksen et al., 2017; Hentschel et al., 2008; Qian et al., 2012; Thakur et al., 2017). Many encapsulation procedures have been proposed, but none of them can be considered universally applicable for bioactive compounds. This is because bioactive compounds in foods have their own molecular structure characteristics (Ramos et al., 2018; Scalbert, Johnson, and Saltmarsh, 2005). Encapsulation should have specific characteristics to withstand environmental influences, protecting the bioactive component from chemical degradation (for example, oxidation or hydrolysis), and maintaining its functionality during the passage through the gastrointestinal tract (Cerqueira et al., 2014; de Vos et al., 2010; Domian, Brynda-Kopytowska, and Oleksza, 2015). Various researchers have tested diverse carrier materials with different physical properties at different temperatures to encapsulate β-carotene (Liang et al., 2013; Lim et al., 2016; Moraes et al., 2013). In general, microencapsulation of carotenoids by spray-drying showed to be very advantageous once microcapsules showed acceptable physical properties and good β-carotene encapsulation efficiencies have been observed (Ahmed et al., 2015; Janiszewska-Turak, 2017). Also, radical scavenging activity studies demonstrated a significant antioxidant activity retention after encapsulation by spray-drying (Munin and Edwards-lévy, 2011).

The choice of wall materials should be based on non-reactivity of the capsule material with the compound to be encapsulated (Favaro-trindade and Pinho, 2008; Ongen et al., 2002). Carbohydrates are frequently used as encapsulating materials due to their absence of flavor or smell, as well as their diversity and low cost. In addition, proteins and lipids have been also used as materials for encapsulating β-carotene (Anal et al., 2015; Souza Simões et al., 2017; Martins et al., 2016). In this context, novel sources of biopolymers are being explored. For example, cereal grains (e.g. corn, wheat and rice) are rich sources of biopolymers with high functionality. Isolated cereal biopolymers (i.e., protein, starch, and non-starch polysaccharides) are often used as thickening ingredients in a variety of food products and more recently, they are being used to produce microparticles for food applications (Joye, 2019). *Amaranthus* spp. (Amaranthaceae family), commonly known as amaranth, is a tropical plant that is still not fully explored. It possesses 60 species, whose plant has a panicle type inflorescence, being frequently classified as a pseudocereal since it does not belong to the grass family and does not contain gluten. Amaranth grain can be used as whole grain, such as whole flour, or combined with other cereals. Some studies explored the use of biopolymers from the amaranth grain as encapsulating materials for bioactive compounds (Coelho et al., 2018). For example, Cortes et al. (2014) used amaranth starch as encapsulating material creating microcapsules with small size (≈ 20 μm) produced by spray-drying to encapsulate probiotics. Also, Kshirsagar and Singhal (2008) showed that amaranth starch is an excellent encapsulating agent for lemon essential oil. Similarly, Aceituno-Medina et al. (2015) have shown that folic acid encapsulation within amaranth protein was a good strategy to decrease folic acid photodegradation and improve its availability. Aceituno-Medina et al. (2015) also showed that the use of amaranth protein in as encapsulation material is advantageous to improve bioactive compounds (quercetin and ferulic acid) antioxidant capacity. Soto et al. (2019) used amaranth protein and pullulan to load polypeptide nisin, reducing its interaction with food and maintaining its antimicrobial activity.

Many studies reported β-carotene encapsulation in various bio-based systems. However, none of them used grain biopolymers that still need to be explored, such as amaranth grain, which has no gluten and has a wide range of nutrients and phytonutrients. Therefore, in this work, both starch and protein from amaranth grain were used to develop β-carotene microcapsules through the spray-drying process and their influence on microcapsules' moisture content, morphology, solubility, particle size, encapsulation efficiency, and β-carotene stability was studied.

3.2 MATERIALS AND METHODS

3.2.1 Extraction of starch and protein from amaranth grain

Amaranth (*Amaranthus cruentus*) grains originating from Peru were purchased and stored in a cold chamber before use.

Starch extraction was performed according to the alkaline wet grinding method described by Cortés *et al.* (2014). Amaranth seeds (100 g) were immersed in 200 mL of 0.1 mol/L NaOH solution (Sigma-Aldrich) for 24 h. Afterwards, the solution was decanted, the seeds were washed with distilled water and further milled in a blender with 150 mL of 0.1 mol/L sodium bisulfite ($NaHSO_3$) (Sigma-Aldrich) cold solution. The suspension was sieved through standard U.S. sieves (841, 420, and 300 μm) to remove the fiber fraction. The starch was isolated using a centrifuge (Sigma 4K15) at 4,226 rpm for 20 min. The supernatant was discarded, and the softer textured upper layer of protein was collected with a spatula. The white starch layer was resuspended in distilled water and centrifuged five more times under the same conditions mentioned above. Both recovered amaranth starch and protein were dried in an oven at 40 °C for 48 h.

Moisture content was determined by measuring the weight loss at 105 °C until constant weight. Ashes were determined by calcination in a muffle at 550 °C. Total nitrogen was determined by the Kjeldahl method (N x 6.25). All the procedures were conducted in accordance with Association of Official Analytical Chemists (Chemists. and Helrich, 1990). Dietary fibers were detected by enzyme-gravimetric method (Chemists. and Helrich, 1990). The samples were analized in triplicate.

The yield of starch and protein extracted from amaranth grain was estimated according to Eq. 3-1:

$$Yield\ \% = \frac{Starch\ or\ protein\ extracted\ (g)}{Inicial\ sample\ quantity\ (g)}\ x\ 100 \qquad \textit{Equation 3-1}$$

3.2.2 Microencapsulation of β-carotene by spray-drying

β-carotene was obtained from Fluka Analytical and corn oil (Fula, Portugal) was purchased in a local store. Prior to spray-drying, β-carotene was dispersed in corn oil (1 % w/w) and added to amaranth starch (0.3 g/mL) or protein (0.3 g/mL) solutions. The solution was prepared at 1:10 (polymer:β-carotene ratio) and further homogenized at 12,000 rpm for 8 min in an Ultra Turrax homogenizer (IKA T18). Microencapsulation was performed by spray-drying according to Cortes et al. (2014). The drying conditions used in the spray-drier (Buchi, Mini Spray Dryer, B-191, Switzerland) were as follows: 180 °C of inlet air temperature; 179 °C of outlet air temperature; 0.5 mm of nozzle diameter; 3 mL/min of liquid flow rate. After the process, the powdered microcapsules were collected and stored in dark at -20 °C until further use.

3.2.3 Encapsulation efficiency of β-carotene in microcapsules

β-carotene was extracted from the biopolimeric microcapsules using ethanol (Sigma-Aldrich) solution as follows: 0.1 g of the sample was placed in 4 mL of ethanol, stirred for 15 s, and then stored in the dark during 15 min, according to Jarunglumlert and Nakagawa method (2013). After centrifugation for 1 min and 12,000 rpm (Sigma 4K15 centrifuge), the supernatant was collected, and the absorption intensity was measured at 450 nm using a UV-Vis spectrophotometer (Jasco V560, USA). The amount of β-carotene extracted in the initial 15 s was used to estimate β-carotene on the microcapsules' surface. The amount of β-carotene obtained after extraction over 24 h was used to evaluate the total encapsulation efficiencies (Jarunglumlert and Nakagawa, 2013). Measurements were performed in triplicate. The encapsulation efficiencies were calculated using Eq. 3-2 and Eq. 3-3:

$$Total\ encapsulation\ efficiency\ = \frac{\text{Extracted quantity of } \beta\text{−carotene after 24 hours}}{\text{Total } \beta\text{−carotene loading in the original solution}} \text{x} 100$$

Equation 3-2

$$Internal\ encapsulation\ efficiency\ = \frac{Total\ extracted\ amount\ of\ \beta-carotene\ -\ surface\ \beta-carotene}{Total\ \beta-carotene\ loaded\ in\ the\ original\ solution}\ x\ 100$$

Equation 3-3

3.2.4 Solubility, moisture content, and water activity of the microcapsules

Singh and Singh (2003) method was used to analyze the water solubility of microcapsule samples. 0.5 g of microcapsules was mixed with 50 ml of water using a magnetic stirrer at room temperature for 30 min. An aliquote of 10 mL of starch or protein microcapsule solution was transferred to a 15 mL tube and centrifuged (Sigma 4K15) at 3,000 rpm during 15 min. An aliquot of 5 mL of the supernatant was evaporated on a steam bath and dried in an oven at 110 °C overnight. The samples' solubility was calculated according to Eq. 3-4:

$$\%\ solubility = \frac{g\ supernatant}{g\ of\ sample}\ x\ 100 \qquad \textit{Equation 3-4}$$

The moisture content (MC) was determined gravimetrically by drying samples (50 mg) at 105 °C in an oven with forced air circulation for 24 h until the equilibrium weight was attained (Chemists. and Helrich, 1990).

The water activity (a_w) was measured using an Aqualab 3 analyzer (Decagon Devices, USA) at 25 °C, after stabilizing the samples at this temperature for 1 h, in triplicate.

3.2.5 Size measurement of microcapsules

To determine the microcapsules' size, a laser diffraction particle size analyzer (Beckman Coulter LS 230, USA) was used, being determined the mean diameter (% volume) and particle size distribution. Measurements were performed in triplicate.

3.2.6 Microcapsules morphology

Microcapsules morphology was studied on an epifluorescence microscope (Olympus BX51) coupled to a DP71 digital camera and three filter sets (DAPI - 360-370/420 nm; FITC - 470-490/520 nm and TRITC - 530-550/590 nm). All images were acquired using Olympus cellSens software Nikon (Eclipse E800, Japan) with 40x and 100x lenses.

Microparticles' structure was also examined in a high-resolution SEM using a SU-70 Hitachi microscope. The acceleration voltage was maintained at 4 kV to avoid sample destruction. Microcapsules were observed on an aluminum support using carbon conductive tape as an adhesive. The samples were coated with Au/Pd conductive coating using a vacuum evaporator.

3.2.7 Fourier-transform infrared spectroscopy (FTIR) measurements

FT-IR spectra of the β-carotene, starch and protein powders, and microcapsules with β-carotene were obtained in a Perkin Elmer Spectrum BX spectrometer using a Golden Gate single reflection diamond ATR system. Spectra were recorded at the absorbance mode from 4,000 to 600 cm^{-1} (mid infrared region) at a resolution of 8 cm^{-1}. Five replicates (128 co-added scans) were collected for each sample (Nunes et al., 2013).

3.2.8 Stability of encapsulated β-carotene

Starch and protein-based microcapsules with β-carotene were stored under different conditions for 90 days: a) at 37 °C in the dark; b) at 25 °C in the dark; c) at 25 °C under lighting conditions; and d) at 8 °C in the dark. Samples were collected every 15 days and analyzed as described below.

3.2.8.1 Determination of β-carotene encapsulated by HPLC

β-carotene content was determined using a standard curve created from a series of β-carotene solutions from 0.03 to 10 μg/mL. After β-carotene dilution in hexane (1.0 mL), HPLC

was used to determine its concentration at wavelength of 455 nm (Zhu et al., 2010). The HPLC equipment consisted of two LC-10AT pumps, a C18 column (Beckman), coupled with a guard column, to analyze the samples at 25 °C. Methanol was used as the mobile phase, being the flow rate 1.0 mL/min.

3.2.8.2 Measurement of the microcapsules color

Microcapsules color was evaluated using a portable colorimeter (Konica Minolta, CR-400), and the CIELAB color space coordinates (L^*, a^* and b^*) were determined with a standard CIE D65 illuminant. The colorimeter was calibrated against a standard white reference. Samples were placed in a white plate and color measurements were performed in triplicate.

The brightness value, L^*, indicates the darkness/luminosity of the sample (L^* varies from 0 (black) to 100 (white); a^* is a measure of greenness/redness of the sample (a^* varies from -80 to +100), and b^* is the extension of blue/yellow (b^* ranges from -50 to +70). The color parameters were measured immediately after spray-drying and were determined periodically during storage (90 days).

The total color difference (ΔE^*) was studied using the Hunter-Scotield equation (Eq. 3-5) (Minolta, 1994):

$$\Delta E* = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2} \qquad \textit{Equation 3-5}$$

The polar coordinate saturation (C^*) was also determined and it indicates the opacity/livability of the microcapsules (C^* varies from 0 to 60) (Eq. 3-6):

$$C* = \sqrt{(a*)^2 + (b*)^2} \qquad \textit{Equation 3-6}$$

The a^* and b^* color coordinates were used to calculate the angle tonality (H^*) (Eq. 3-7). The angles between 0° and 90° characterize the color quality and vary from red to yellow. Those angles that are closer to 90° (yellow) are indicative of greater loss of β-carotene.

$$H* = tan^{-1}\left(\frac{b*}{a*}\right) \qquad \textit{Equation 3-7}$$

3.2.9 Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided *t*-tests (means test of equality) at the 95% significance level ($p < 0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction of starch and protein from amaranth grain

The starch extraction yield was 32.3 ± 0.2% and the protein extraction yield was 13.1% ± 0.2. The yield of protein and starch extractions was superior to those reported by Villareal et al. (2013), Uriyapongson and Rayas-Duarte (1994) and Malinski et al., (2003). These results may be due to the fact that the grain is ground before alkaline treatment which favored protein release. (Bello-Pérez et al., 1998; Kong et al., 2010; Radosavljevic et al., 1998) showed starch extraction yields of 44.4%, 34.8% and 30.4%, respectively, similar to our study, suggesting the appropriatness of the method used to extract starch and protein from amaranth.

Table 3-1 shows the amount of starch and protein extracted from amaranth, higher than previously reported in literature (Myers and Fox, 1994; Burisová et al., 2001). Probably in our work this may be due to the larger and heavier amaranth fractions used, the repeated washing, filtration steps, and centrifugation that were performed which reduced the ash and fiber content in the final extraction. (Uriyapongson and Rayas-Duarte, 1994) obtained lower amounts of protein and fiber in starch extraction than the values presented in this paper, possibly due to the previous abrasive separation of protein-fiber that improved subsequent starch extraction.

Table 3-1- Centesimal analysis of starch or protein extracted from amaranth

	Amaranth Starch	**Amaranth Protein**
Ashes (g/100g)	1.30 ± 0.01	0.80 ± 0.02
Protein (g/100g)	1.90 ± 0.02	16.5 ± 0.02
Dietary Fiber (g/100g)	2.60 ± 0.05	0.85 ± 0.02

3.3.2 Encapsulation efficiency of β-carotene in microcapsules

Maximum encapsulation efficiency is desirable for any formulation. This parameter not only helps preventing the loss of biological action of photosensitive materials, but also helps extending the action time of the bioactive compound. The encapsulation efficiency is important for the retention of bioactive compound, mainly because it is usually a thermolabile and photosensitive compound (Desobry, Netto, and Labuza, 1997).

The total amount of β-carotene encapsulated in starch and protein microcapsules was 10 mg/L. The total β-carotene internal encapsulation efficiency was 34.35 ± 1.95 % for starch-based microcapsules and 26.71 ± 0.42 % for protein-based microcapsules. The encapsulation efficiency was 68.62 ±0.28 % for starch-based microcapsules and 64.09 ±0.33 % for protein-based microcapsules. The β-carotene encapsulation efficiencies results showed no significant differences between the two biopolymers used (p>0.05). The β-carotene encapsulation efficiencies obtained in our work were very similar to the encapsulation efficiencies values described in other studies: Rostamabadi et al., (2019) used glucuronoxylan, Jiang et al., (2017) used whey protein isolate and galactose; and Thakur et al. (2017) used casein/guar gum. For instance, Morowvat and Ghasemi (2016) obtained an β-carotene encapsulation efficiency of 93.22% using microalgae biomass, and was able to preserve the β-carotene from thermal degradation during the spray drying procedure. In other work, Donhowe et al. (2014) obtained a much lower encapsulation efficiencies - 37.7% and 54.7% - for microcapsules with maltodextrin and chitosan, respectively.

Some researchers (Kha et al., 2014; Mohammed et al., 2017; Murali et al., 2016) reported that the concentration of wall material has a positive effect on encapsulation efficiency, as increasing the solids content in the capsules increases bioactive retention. This behavior may be related to the reduction of the time required to form the surface crust on the atomized droplets in

the initial drying process when the solids concentration in the feed solution increases. The rapidly formed crust is not permeable to compounds, thus protecting the bioactive from oxidation (Correâ-Filho et al., 2019).

3.3.3 Microcapsules solubility, moisture content, and water activity

The water solubility of the microcapsule wall material is a very important feature, because the water is the most used solvent in the food industry, and solubility is related to the integrity of the microcapsule wall that causes the release of the active compound into the medium (Spada et al., 2012).

The solubility in cold water of protein and starch microcapsules with β-carotene showed significant differences ($p < 0.05$) (Table 3-2). Amaranth starch microcapsules showed higher solubility in cold water than protein microcapsules. Probably, these results are due to differences in the biopolymers solubility capacity. It is noteworthy that the starch solubility varies with the botanical source, as starch molecules interaction with water is influenced by amylose:amylopectin ratio and the characteristics of these molecules such as molecular weight distribution, degree and length of branches and conformation (Singh and Singh, 2003). This result was already expected and there was a statistical difference ($p <0.05$) in the solubility between starch and protein capsules, since starch is more water soluble, thus facilitating its incorporation in aqueous systems, also performing gelatinization if necessary (Liu et al., 2019).

Table 3-2 - Solubility, water activity, and moisture content of amaranth protein and starch-based microcapsules with β-carotene.

Type of Biopolymer	Solubility (%)	Water activity	Moisture content (%)
Protein	9.28 ± 0.02[a]	0.30 ± 0.01[a]	6.2 ± 0.03[a]
Starch	25.08 ± 0.01[b]	0.21 ± 0.00[a]	5.7 ± 0.02[a]

Mean of three replicates ± standard error.

[a,b] Different superscript letters in the same column indicate significant differences between samples ($p < 0.05$)

Water activity is an important feature that directly affects biochemistry and microbiological changes, as well as food products shelf-life. A product with low water activity can be safely stored without refrigeration. Foods with water activities above 0.6 are at risk of growth and microbial spoilage (Ngamekaue and Chitprasert, 2019). As shown in Table 2, water activity was less than 0.6 thus, offering a good shelf-life and producing a powder without agglomeration (Fioramonti et al., 2019). All values were within the range of 0.2 to 0.3, which is considered stable for lipid oxidation, microbial growth, darkening, and enzymatic reactions (Caliskan and Dirim, 2016). Similar results were observed when β-carotene was encapsulated in other starch-based and protein-based matrices (Jain et al., 2016; Loksuwan, 2007; Thakur et al., 2017).

3.3.4 Size of biopolymer-based microcapsules produced by spray-drying

In general, the two types of microcapsules produced by spray drying presented a bimodal size distribution, a characteristic that occurs due to the microencapsulation process employed (Kong et al., 2008).

The average size of the microcapsules composed of protein or starch of amaranth was 2.22 ± 1.84 μm and 1.55 ± 1.12 μm, respectively (Figures 3-1 and 3-2). The standard deviations were high, since the protein-based and starch-based microcapsules diameters ranged from 0.3 to 30 μm and 0.3 to 15 μm, respectively, and a peak of agglomeration was present in both cases. Prossibly, smaller size particles could be encapsulating material (i.e. starch or protein) that failed to encapsulate β-carotene. On the other hand, higher size values may be possibly due to microcapsule agglomeration, also observed in the microscopy images (Figures 4 and 5), where the formation of bonds between them could have occured (Tonon, Brabet, and Hubinger, 2010). Moreover, several parameters could influence the microparticles' average size obtained by spray-drying, being the process parameters used (e.g., spray-drying pressure, feed rate, carrier material, solution viscosity and spray-drier size) determining factors (Versic, 2014). Particle sizes obtained by spray-drying as encapsulating technique are, in general, within the range of particle sizes produced by other techniques such as coacervation, which is 5–150 μm (Spada et al., 2012; Yeo and Park, 2004).

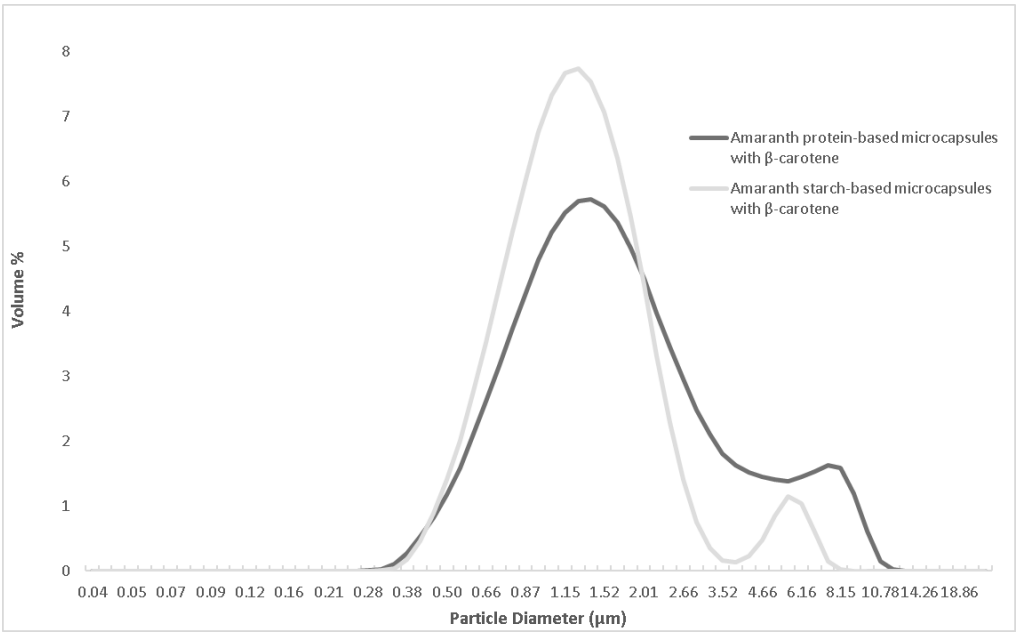

Figure 3-1 - Particle size distribution of amaranth protein and starch-based microcapsules with β-carotene.

Particle size distribution is a physical powders parameter which may infuence their properties involving handling, transport, and storage such as bulk density, angle of repose, fowability, rehydration capacity, solubility, and dispersibility (Akhavan et al., 2016). According to (Tontul and Topuz, 2017) the stability of the sensitive functional components to environmental conditions is also afected by the particle size and the liquid droplet size during atomization which varies directly with the liquid viscosity at constant atomizer speed, resulting in larger particles. According to several authors (Correâ-Filho et al., 2019; Gul et al., 2015; Ordoñez-Santos, Martínez-Girón, and Villamizar-Vargas, 2018; Paim et al., 2016), slower drying rate, i.e., when the inlet drying temperature is low, the particles shrink evenly, making their size smaller. However, when the drying rate is higher, the rapid evaporation of the water creates a hard crust in the particle that prevents its contraction in the drying process, resulting in larger particles.

3.3.5 Morphology of spray-dried microcapsules

Figure 3-2 shows that β-carotene was effectively encapsulated due to the orange color

within the microcapsules.

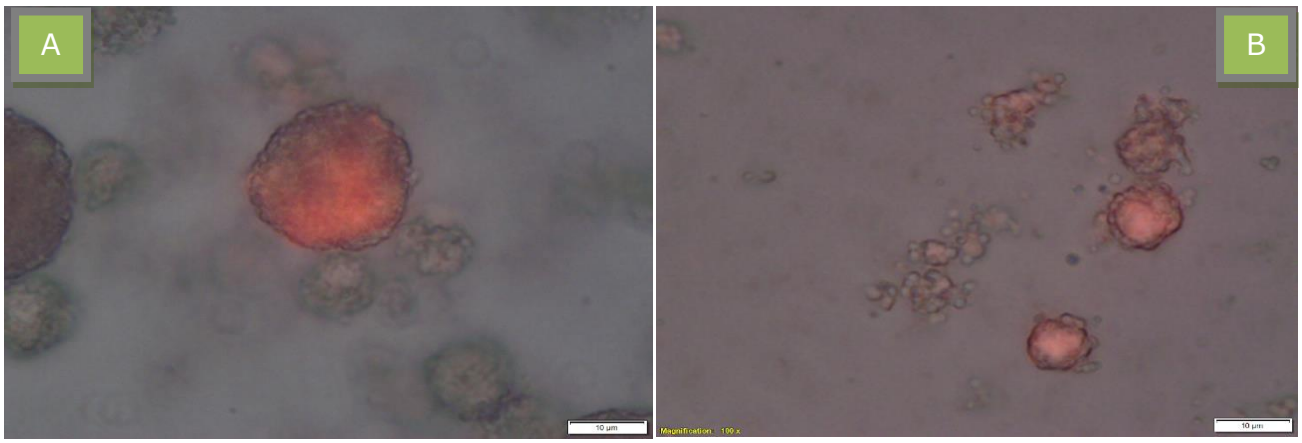

Figure 3-2 - Fluorescence micrographs of the β-carotene encapsulated on protein (A) and starch (B) microcapsules, obtained with a magnification of 100x.

In the SEM micrographs shown in Figure 3-3, it is possible to verify the presence of spheric-like structures, apparently resulting from the aggregation of particles. The amaranth starch structures seem to be slightly larger than the amaranth protein ones. The round-shaped structures of the amaranth protein-based microcapsules look like being completely closed with very compact aggregates of particles. The amaranth starch-based microcapsules resemble to have small interstices between the particles (which are better defined). The interstices look like giving access to the hollow part of the spheres. The presence of voids at the interior of the spherical aggregates may explain the good capacity of amaranth starch and protein to encapsulate β-carotene (Leimann, 2008). Moreover, microcapsules size variability and clustering occur, together with some surface imperfections (Figure 3-3), which are possibly due to particle surface crust formed during drying of the spray droplets (Rosenberg, Kopelman, and Talmon, 2006). Typically, it is desirable that the aggregates of particles are spherical in order to enhance the capacity to encapsulate compounds. According to (Osorio et al., 2010), smooth capsules are desirable for encapsulated compound stability and controlled release.

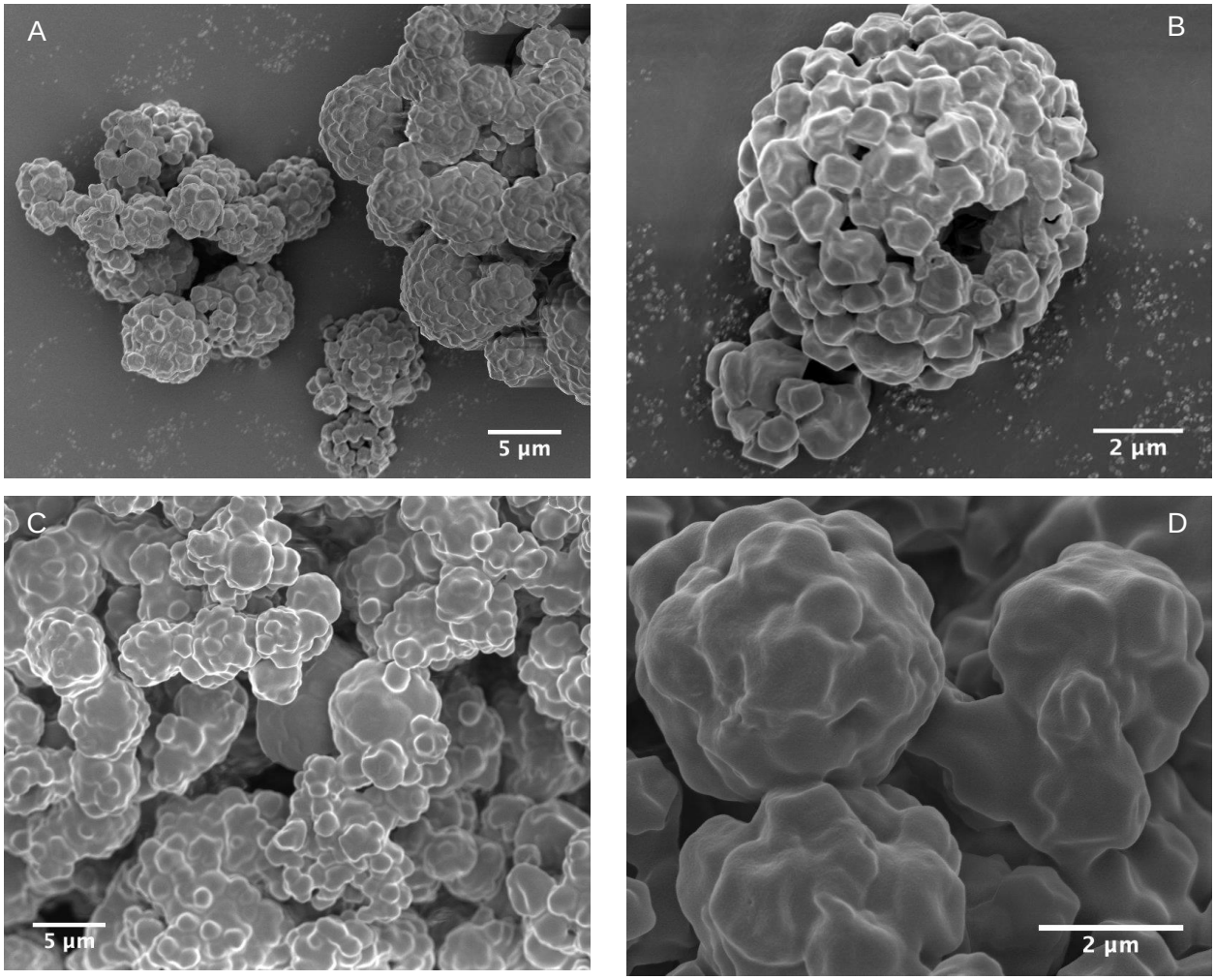

Figure 3-3 – SEM micrographs of amaranth starch-based microcapsules with β-carotene (A and B) and amaranth protein-based microcapsules with β-carotene (C and D).

Starch-based capsules present a porous-like surface with spaces between particles, while the protein capsules seem to be more compact as shown in Figure 3-3. This may influence the drying rate and consequently, the solubility properties studied.

3.3.6 Infrared spectroscopy (FT-IR)

FTIR spectroscopy was used to chemically analyze the protein and starch microcapsules with β-carotene. In addition, protein and starch powders and β-carotene in carrier oil spectra were obtained for comparison purposes (Figure 3-4).

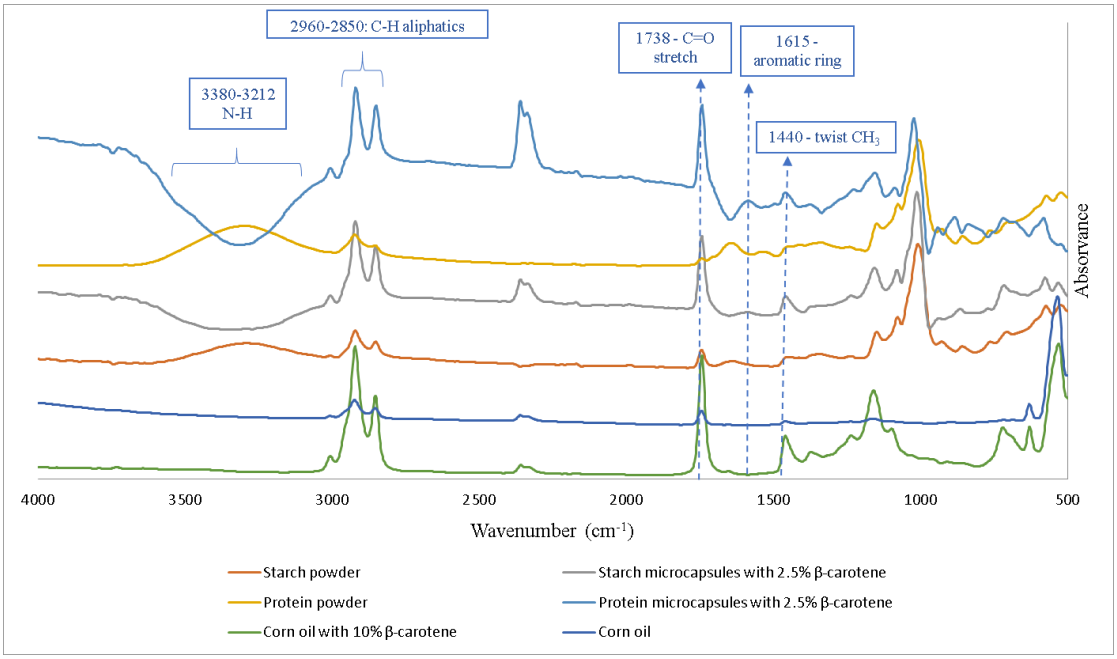

Figure 3-4 – ATR-FTIR spectra of corn oil, β-carotene in corn oil, starch and protein powders and microcapsules loaded with β-carotene. Typical FTIR spectrum peaks of β-carotene are indicated.

According to (Moh et al., 1999), β-carotene absorption bands can be observed at 956, 1373, 1455, and 3005 cm^{-1}. Therefore, microcapsules loaded with β-carotene spectra exhibited peaks corresponding to 3005 cm^{-1}, confirming the presence of the stretchable OH bond and exhibited distinct peaks at 1455 cm^{-1}, which is the CH elongation in the aromatic ring. We can also observe a bulging at 3308 cm^{-1}, which differs from the β-carotene spectrum, which occurs mainly in protein microcapsules because it is the spectrum region of the amines.

The β-carotene loaded capsules showed a considerable increase in the absorbance of the bands between 2960 and 2850 cm^{-1} comparing to starch and protein powders where the C-H aliphatic bonds are found (Gómez-Mascaraque et al., 2017). At 1440 cm^{-1} peak was also found in the loaded structures. This band can be attributed to the contribution of CH_3 bonds found in carotenoids, and consequently, β-carotene (Rubio-Diaz, Francis, and Rodriguez-Saona, 2011).

The band caused by C=O group stretching occurred at 1738 cm^{-1}, increased in the β-carotene microcapsules. However, protein and starch powders spectra also showed this band at a smaller angle, making it difficult to attribute them only to the presence of β-carotene. This, coupled

with the low β-carotene concentration in the capsules, made it difficult to unambiguously identify β-carotene in the FTIR spectra of the materials, as well as to evaluate their possible interactions with starch and protein.

3.3.7 Stability of encapsulated β-carotene

Figure 3-5 shows the evolution of β-carotene concentration when incorporated into starch or protein microcapsules during storage under different storage conditions, paired samples test there was significant difference between all means ($p < 0.05$) compared to each other. It has been reported that the degradation of β-carotene is mainly attributed to oxidation (Knockaert et al., 2012). Overall, it can be seen that β-carotene stability within the protein microcapsules was higher than within starch microcapsules during 90 days at 8 and 25 °C (Figure 3-5), confirmed by the statistical analysis of the data ($p < 0.05$).

The improved β-carotene thermal stability within the protein microcapsule can result from several reasons. Firstly, the protein has a larger retention network that can act as a physical barrier that isolated and protected the compound from external factors (such as temperature) and prevented any pro-oxidants in the aqueous phase to contact with β-carotene. Secondly, amaranth protein has cysteine residues and other functional groups that can serve as antioxidants, either by chelating transition metals or by acting as free radical scavengers (Singh et al., 2019).

Thirdly, the protein can form molecular complexes with β-carotene through hydrophobic interactions or van der Waals interactions that may help protect the β-carotene molecules within the microcapsule (Qian et al., 2012). Dianawati et al. (2017) demonstrated that the addition of proteins as encapsulating materials had a significantly higher protective effect compared to the control sample, which also occurred after spray drying and during storage at different temperatures. In addition to the protein having higher emulsifying properties and lower viscosity leading to lower shear stress and resulting longer term protection, it facilitates the formation of dense gel mesh microcapsules that provide substantial buffering capacity, thus supporting the idea of a protective barrier between sensitive core material and the surrounding environment (Heidebach, Först, and Kulozik, 2012). Amaranth protein has cysteine residues and other functional groups that can serve as antioxidants, either by chelating transition metals or by acting

as free radical scavengers (Singh et al., 2019). In addition to the major role of β-carotene as a nutrient source of vitamin A, β -carotene also has antioxidant capacity and can function as a lipid radical scavenger and a singlet oxygen suppressor due to the unique structure of conjugated double bonds and ionone rings (Donhowe, 2010).

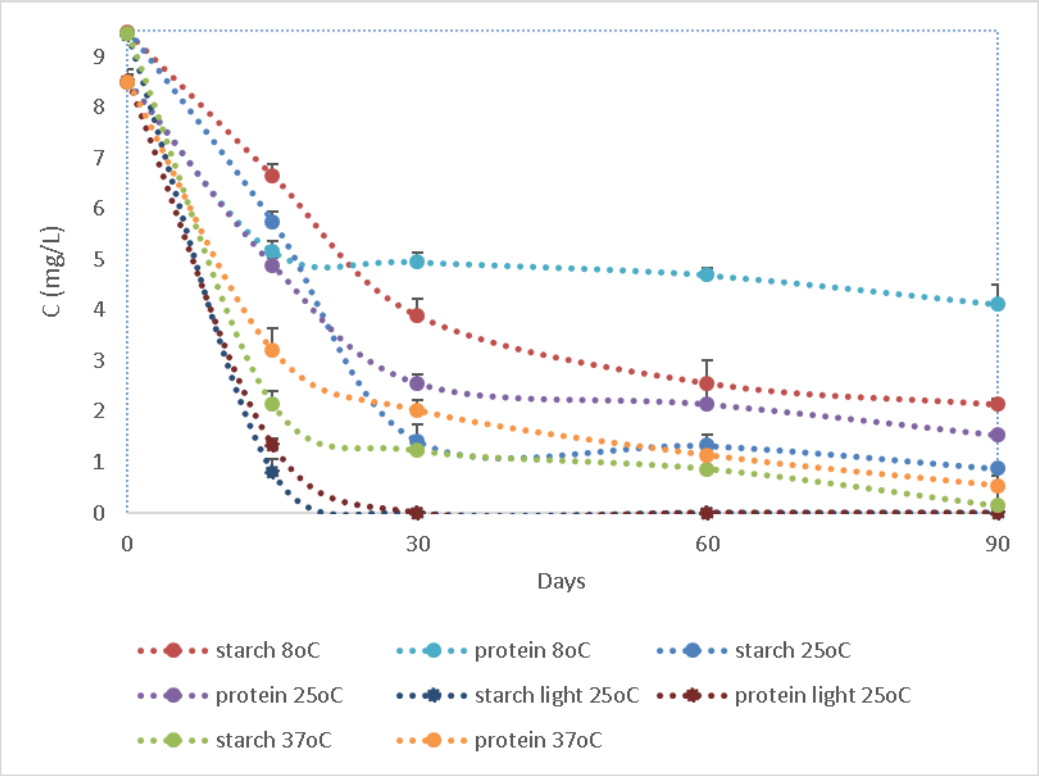

Figure 3-5 - Stability of β-carotene incorporated into microcapsules of amaranth starch or protein during 90 days of storage at 8, 25 and 37 °C under dark conditions and 25 °C under ligh conditions.

Moreover, Spada et al. (2012) found that the loss of β-carotene encapsulated in starch capules in the presence of light was 90%, in less than 30 days. (Rocha-Selmi, Favaro-Trindade, and Grosso, 2013) also observed an effect of light on carotenoid degradation. These authors encapsulated carotenoids in gum arabic and maltodextrin by lyophilization and obtained better stability for samples stored in the dark than under light at room temperature. Also, β-carotene degradation increases as temperature increases. (Desobry et al., 1997) encapsulated β-carotene using maltodextrin and studied the stability of these capsules at 25, 35 and 45 °C. The authors

concluded that 80% of β-carotene degraded after 7 weeks at 45 °C and after 12 weeks at 35 °C. In addition, after 12 weeks at 25 °C, the retention rate of β-carotene was equal to 30%.

3.3.8 Measurement of microcapsules color

Pure β-carotene has a dark red color, while amaranth starch presents a white color and amaranth protein is yellowish white. In fact, starch and protein microcapsules have initial L^* values of 90.68 ± 0.02 and 77.94 ± 0.04, a^* values of -0.26 ± 0.01 and 2.43 ± 0.01, and b^* values of 9.84 ± 0.04 and 14.35 ± 0.01, respectively (Table 3-3).

According to Table 3-3, the parameter that best represented the observed color changes was the chromatic coordinate a* (redness). After 90 days of storage, a^* values decreased and L^* values increased for starch microcapsules because β-carotene oxidation reduced the red color intensity of the microcapsules and increased their white color. This loss of color also shows loss of biological activity, and indicates the formation of apocarotenoids. The parameter b^* was not a good indicator of β-carotene retention because it characterizes the extent of yellowness to blueness, and these colors were not dominant. Similar results were reported by Spada et al. (2012) and Sutter, Buera, and Elizalde (2007b). These authors evaluated β-carotene color when encapsulated in maltodextrin systems and they found that L^* (lightness) and a^* (redness) were the most sensitive parameters to study β-carotene color changes. On the other hand, protein microcapsules showed an increase in a^* values and a decrease in L^* values, explained by the fact that the protein increased the β-carotene stability within the microcapsule comparing to starch microcapsules, as shown in Figure 3-5.

The ΔE^* values increased considerably after 90 days of storage, i.e. after storage the samples did not show the same initial color characteristics. It should be noted that the higher the value of ΔE^*, the higher the total color difference of the analyzed sample in relation to the initial sample. The highest ΔE^* values were found for both microcapsule samples exposed to light and 25 °C. On the other hand, the lowest ΔE^* values were observed for samples stored at 8 °C and under dark conditions. As expected, β-carotene oxidation was reduced in the last mentioned case due to reduction of the main oxidative factors (i.e. temperature and light), corroborating the results obtained for other β-carotene microcapsules (Chen et al., 2014; Fu et al., 2019; Mehrad et al.,

2018; Spada, et al., 2012; Sutter et al., 2007b). Sutter, Buera, and Elizalde, (2007a) concluded that the coordinate a^* best represented color changes observed in β-carotene encapsulated in mannitol-phosphate matrix. Other studies have shown a strong relationship between a^* and the total carotenoid concentration of foods (Borba et al., 2019; Liu et al., 2018; Ramakrishnan and Francis, 1973; Takahata, Noda, and Nagata, 1993).

The angle of hue (H^*) and chroma (C^*), which are a combination of coordinates, were also analyzed. These colorimetric parameters are widely used to characterize food color variation during processing. (Gonçalves et al., 2007; Maskan, 2001; Skrede et al., 1997) used H^* to investigate the influence of the chemical structure in the color of the carotenoids. They obtained H^* values from 74.16 to 76.59, similar to the values obtained in our work (Table 3-3). In general, it has been seen that the opening of the end rings or increasing conjugation of carotenoids increases H^* value (Meléndez-Martínez, Britton, Vicario, and Heredia, 2007). Overall, samples in contact with light presented higher values of ΔE^*, H^* and C^*, which is indicative of a higher red color loss and significant changes in the original H^* or tonality (Meléndez-Martínez et al., 2007; Zhang, Zhang, and McClements, 2016).

It can be understood that preservation under dark and cold conditions causes more stability for microencapsulated β-carotene. In our work, the best result was seen at 8 °C under dark conditions in accordance to (Morowvat and Ghasemi, 2016) results. Also, encapsulated β-carotene suffered a progressive loss under light and/or high temperature conditions but exhibited good stability at low temperatures (e.g., 8 C) as described by other authors (Fu et al., 2019).

Table 3-3 - Color of the microcapsules with β-carotene after 90 days of storage under different temperature and light conditions.

Microcapsule	Sample storage condition	Color parameter					
		*L**	*a**	*b**	*ΔE**	*H**	*C**
Protein	Initial (*t*=0)	90.68 ± 0.02[b]	-0.26 ± 0.01[b]	9.84 ± 0.04[b]			
	Dark, 25 °C	77.52 ± 0.01[a]	1.59 ± 0.00[a]	26.94 ± 0.01[a]	12.62 ± 0.03	86.62 ± 0.02[a]	26.99 ± 0.01[a]
	Dark, 8 °C	78.84 ± 0.06[a]	2.46 ± 0.07[a]	13.78 ± 0.07[a]	1.07 ± 0.07	79.88 ± 0.08[a]	14.00 ± 0.07[a]
	Light, 25 °C	53.70 ± 0.08[a]	9.04 ± 0.01[a]	43.55 ± 0.06[a]	38.52 ± 0.06	78.28 ± 0.01[a]	44.48 ± 0.06[a]
	Dark, 37°C	76.52 ± 0.03[a]	1.77 ± 0.02[a]	25.77 ± 0.01[a]	11.52 ± 0.04	86.07 ± 0.03[a]	25.83 ± 0.01[a]
Starch	Initial (*t*=0)	77.94 ± 0.04[b]	2.43 ± 0.01[b]	14.35 ± 0.01[b]			
	Dark, 25 °C	87.60 ± 0.03[a]	-1.27 ± 0.08[a]	35.18 ± 0.05[a]	25.55 ± 0.08	92.08 ± 0.11[a]	35.20 ± 0.05[a]
	Dark, 8 °C	91.89 ± 0.07[a]	-0.04 ± 0.01[a]	6.85 ± 0.07[a]	3.23 ± 0.03	90.67 ± 0.07[a]	6.85 ± 0.07[a]
	Light, 25 °C	85.54 ± 0.10[a]	-0.74 ± 0.06[a]	36.24 ± 0.01[a]	26.90 ± 0.01	91.16 ± 0.09[a]	36.24 ± 0.01[a]
	Dark, 37°C	86.47 ±0.03[a]	-1.19 ± 0.01[a]	34.31 ± 0.07[a]	24.85 ± 0.09	91.98 ± 0.05	34.33 ± 0.08[a]

Mean of three replicates ± standard error.

[a,b] Different superscript letters in the same column indicate significant differences between the initial value (*t*=0 d) and the final value (*t*=90 d) ($p<0.05$)

3.4 CONCLUSION

This study shows that it is possible to produce stable microcapsules encapsulating β-carotene using amaranth starch or protein as wall materials.

All microcapsules showed defined shapes and varied sizes, and these characteristics are related to the process used for their preparation. Based on the analysis of granulometric distribution, it was verified that the produced particles encapsulating β-carotene had a diameter that allows their characterization as microcapsules. All samples showed good solubility in water, moisture content, and water activity, when compared to other similar microcapsules found in the literature. The stability tests showed that β-carotene within microcapsules are sensitive to light.

In conclusion, β-carotene-loaded microcapsules developed using spray-drying and biopolymers extracted from amaranth as wall materials are promising to ensure β-carotene long-term storage stability. The recommended intake of vitamin A (retinol) is 800 equivalents of retinol activity (RAE) (1 RAE = 1 μg retinol = 12 μg β-carotene), therefore, 1.33 g of this microcapsule would be needed to meet vitamin A recommended daily intake.

The microcapsules produced with amaranth polymers meet the current trend for gluten-free products and the full use of foods (such as amaranth grain) that are still under-exploited.

Future research is needed to study the β-carotene bioavailability and release from microcapsules. In addition, it is still necessary to evaluate their behavior when incorporated into food simulants, further studies that we are carrying out.

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CHAPTER 4

IN VITRO RELEASE, BIOACCESSIBILITY AND CYTOTOXICITY OF β-CAROTENE MICROCAPSULES PRODUCED WITH STARCH AND PROTEIN FROM AMARANTH GRAIN

The aims of this study were to determine β-carotene release from amaranth starch and protein microcapsules and their cytotoxicity. The β-carotene bioaccessibility was also assessed using an *in vitro* digestion model.

The results presented in this Chapter were adapted from:

Coelho, L.M., Pinheiro, A., Martins, J., Vicente, A. Bioaccessibility and toxicity of β-carotene microcapsules produced with starch and protein from amaranth grain. *Innovative Food Science and Emerging Technologies* (submitted).

4.1 INTRODUCTION

Carotenoids are highly conjugated fat soluble phytonutrients responsible for the orange, yellow and red colors of fruits and vegetables (Roman, Burri, and Singh, 2012b). More than 600 different carotenoids have been described so far, of which carotene is the most significant (Laos et al., 2007). In both developing and developed countries the efficient β-carotene delivery is needed for optimum health of their populations. Delivery of β-carotene by dietary supplementation is limited by the insolubility of β-carotene in aqueous solutions, including saliva and intestinal fluids, but is facilitated by dietary fat and oils (Yi et al., 2015). In humans, these compounds have preventive effects against major diseases such as carcinomas, arteriosclerosis, cardiovascular disease, macular degeneration, and other age-related disorders (Lin et al., 2018). Carotene has been the subject of extensive research due to its provitamin A activity and antioxidant properties (Jain et al., 2015). Besides, the β-carotene deficiency may result in vision disability in humans and increased mortality due to a weakened innate immunity and adaptive immunity (Saini, Nile, and Park, 2015).

β-carotene bioavailability is generally poor and its absorption may be less than 10 % (Faulks and Southon, 2005; Zakynthinos and Varzakas, 2016), which is mainly attributed to carotene and protein complexes and plant cell walls resistance to achieve adequate β-carotene release (Rein et al., 2013). Its low bioavailability has led to the development of β-carotene delivery systems (Donhowe and Kong, 2014). Also, pure β-carotene is easy to degrade due to its sensitivity to oxygen, light and temperature. Encapsulating β-carotene in suitable delivery vehicles is a useful method to protect β-carotene from degradation.

β-carotene microencapsulation is a method for improving storage stability and perhaps increase its controlled release and bioaccessibility during digestion (Roman, Burri, and Singh, 2012a) and this is a versatile and widely used technical feature for encapsulating sensitive ingredients within a wall or coating material. Coating materials ensure the protection of core sensitive ingredients against adverse reactions, preserve volatility and core material controlled release (Risch, 1995; Shahidi, 2009). Microencapsulation has established numerous applications in the pharmaceutical, food, agricultural, pesticide, cosmetic, textile and other related fields. The most imperative applications are the encapsulation of pigments, flavors, vitamins and other sensitive ingredients in order to increase their shelf life (Thakur et al., 2017).

Natural biopolymers, due to their ecological nature, free access, good film-forming properties, cost-effectiveness and most importantly their biodegradable nature, are undoubtedly the best choice for microcapsule wall material. Different studies have shown that biopolymers extracted from amaranth grain can be used as encapsulating materials. For example, small starch granules from *Amaranthus paniculatus* in combination with polysaccharide bonding agents (i.e., gum arabic, carboxymethyl cellulose and carrageenan) were used for the entrapment of a flavoring compound, vanillin. It was shown that gum arabic with 1.0 % amaranth starch gave better vanillin encapsulation results than carboxymethylcellulose and carrageenan (Tari et al., 2003). Amaranth starch was a better encapsulation material than starch extracted from quinoa, rice and colocasia, which was explained by the amylose quantity variation in the each grain (Raul Avila-Sosa et al., 2012; Cortés et al., 2014).

Bioactive compounds encapsulation, namely β-carotene, within amaranth-based capsules could be considered as one approach to enhance compounds bioavailability and to protect them during their passage through the GI tract and consequently to deliver the compound at a particular point, thus taking full advantage of its beneficial effect (Coelho et al., 2018). To evaluate this, usually *in vitro* digestion models are used to predict β-carotene bioavailability by measuring β-carotene content in micellar phase (Rodriguez-amaya, 2010). In addition to being more economical, easier, and more reproducible than human trials, *in vitro* models have been validated due to their strong correlation with *in vivo* results (Granado-Lorencio et al., 2017). Preserving β-carotene functionality is dependent on the physical properties after microencapsulation. Decreasing surface β-carotene content, for example, increases retention by directly decreasing the amount of β-carotene exposed to oxygen and with a higher risk of oxidation (Desobry, Netto, and Labuza, 1998).

Spray-dried β-carotene preservation and shelf life stability was achieved by other authors (Donhowe, 2010; Loksuwan, 2007), but β-carotene release from amaranth starch or protein microcapsules and its bioaccessibility during *in vitro* digestion has not been studied yet. Chitosan-alginate β-carotene microcapsules developed for enteric release have been studied during an *in vitro* digestion model (Roman et al., 2012a).

The objectives of this work were to study β-carotene release from microcapsules developed in Chapter 3 and its bioaccessibility (i.e. amount incorporated into the micellar phase) during *in vitro* digestion. Also, its cytotoxicity and cellular antioxidant capacity on Caco-2 cell cultures were assessed.

4.2 MATERIALS AND METHODS

4.2.1 Extraction of starch and protein from amaranth grain

Amaranth (*Amaranthus cruentus*) grains originating from Peru were purchased and stored in a cold chamber before use, β-carotene (Fluka Analytical) and corn oil (Fula).

Starch extraction was performed according to the alkaline wet grinding method described by (Cortés et al., 2014). Amaranth seeds (100 g) were immersed in 200 mL of 0.1 mol/L NaOH solution for 24 h. Afterwards, the solution was decanted, the seeds were washed with distilled water and further milled in a blender with 150 mL of 0.1 mol/L sodium bisulfate ($NaHSO_3$) cold solution. The suspension was sieved through standard U.S. sieves (841, 420 and 300 μm) to remove the fiber fraction. The starch was isolated using a centrifuge (Sigma 4K15) at 4,226 rpm for 20 min. The supernatant was discarded, and the softer textured upper layer of protein was collected with a spatula. The white starch layer was resuspended in distilled water and centrifuged five more times under the same conditions mentioned above. Both recovered amaranth starch and protein were dried in an oven at 40°C for 48 h.

4.2.2 Microencapsulation of β-carotene by spray-drying

Prior to spray-drying, β-carotene was dispersed in corn oil (1 % w/w) and added to amaranth starch (0.3 g/mL) or protein (0.3 g/mL) solutions. The solution was prepared in 1:10 (polymer:β-carotene ratio) and further homogenized at 12,000 rpm for 8 min in an Ultra Turrax homogenizer (IKA T18). Microencapsulation was performed by spray-drying according to other atutors (Cortés et al., 2014). The drying conditions used in the spray-drier (Buchi, Mini Spray Dryer, B-191, Switzerland) were as follows: 180°C of inlet air temperature; 179°C of outlet air temperature; 0.5 mm of nozzle diameter; 3 mL/min of liquid flow rate. After the process, the powdered microcapsules were collected and stored in dark at -20°C until further use.

4.2.3 *In vitro* β-carotene release studies

β-carotene microcapsules were subjected to *in vitro* release studies using four different receptor media - 20 % (v/v) ethanol, 5 % (v/v) sodium dodecyl sulfate (SDS), phosphate buffered saline (PBS) (with 5 % Tween), and HCl 0.1 N (with 5 % Tween) – using a dialysis membrane diffusion technique (Cerqueira et al., 2014). The HCl solution was used to simulate stomach pH conditions (pH = 2). Ethanol solution is a food simulator that has a hydrophilic character and is capable of extracting hydrophilic substances. Ethanol should be used as a simulator for alcoholic foods with an alcohol content of up to 20 % (v/v) and for foods containing a relevant amount of ingredients that make the food more lipophilic (Cerqueira, Pinheiro, et al., 2014). SDS is an anionic detergent and β-carotene can be solubilized in SDS aqueous solution (Szymula, 2004). PBS (pH=7.4) is commonly used to mimic colon and small intestine conditions (Simões et al., 2020).

For this study, 1 g of the microcapsules were placed into a dialysis membrane (1.5 ml) (MWCO 10-12 kDa), hermetically sealed and immersed in 50 mL receptor medium. The medium was agitated (100 rpm) and the tests were performed at 37 ± 1 °C. The receptor compartment volume was kept stable by replenishing the medium with 1 mL receptor solution after each 1 mL of sample withdrawal. The sample collected was analyzed by UV-VIS spectrophotometry (Jasco V560, USA) at 450 nm (absorbance peak). β-carotene concentration was determined from a previously prepared calibration curve of absorbance versus β-carotene (0.8 to 10 mg/L).

4.2.4 *In vitro* digestion and β-carotene bioaccessibility determination

4.2.4.1 Materials

α-amylase, Porcine pepsin, pancreatin (8 × USP), bile extract porcine, HCl, $NaHCO_3$, $(NH_4)_2.CO_3$ and pefabloc SC (4-(2-aminoetyl) benenesulfonyl fluoride) were purchased from Sigma-Aldrich (St. Louis, MO). NaOH, KCl, $CaCl_2.(H_2O)_2$, KH_2PO_4 and NaCl were obtained from Panreac (Spain) and $MgCl_2.(H_2O)_6$ were purchased from Merck. Chloroform was obtained from Fisher Scientific (NJ, USA).

4.2.4.2 In vitro digestion

In vitro digestion was performed as described by other authors Minekus et al (2014). Briefly, 1 g of microcapsules suspension in 5 mL water was subsequently exposed to conditions simulating the environment of the mouth, stomach and small intestine.

The simulation of the oral phase consisted in the addition of α-amylase (to obtain an activity of 75 U/mL), simulated salivary fluid (SSF) (KCl 15.1 $mM.L^{-1}$, KH_2PO_4 3.7 $mM.L^{-1}$, $NaHCO_3$ 13.6 $mM.L^{-1}$, $MgCl_2.(H_2O)_6$ 0.15 $mM.L^{-1}$, $(NH_4)_2.CO_3$ 0.06 $mM.L^{-1}$, and HCl 1.1 $mM.L^{-1}$), $CaCl_2.(H_2O)_2$ (to obtain 1.5 $mM.L^{-1}$ in the fluid) and purified water (necessary volume to dilute the SSF stock solution). The mixture was incubated for 2 min at 37 °C.

The simulation of the gastric phase has been performed by adding the gastric secretion consisting of porcine pepsin solution (2000 $U.mL^{-1}$ in the final mixture), simulated gastric fluid (SGF) (KCl 6.9 $mM.L^{-1}$, KH_2PO_4 0.9 $mM.L^{-1}$, $NaHCO_3$ 25 $mM.L^{-1}$, NaCl 47.2 $mM.L^{-1}$, $MgCl_2.(H_2O)_6$ 0.1 $mM.L^{-1}$, $(NH_4)_2.CO_3$ 0.5 $mM.L^{-1}$ and HCl 15.6 $mM.L^{-1}$), $CaCl_2.(H_2O)_2$ (to obtain 0.15 $mM.L^{-1}$ in the fluid), HCl to adjust pH to 3.0 and purified water (volume needed to dilute the SGF stock solution). Then, the samples were incubated in a shaking bath at 37 °C for 2 h.

The intestinal phase has been simulated by adding simulated intestinal fluid (SIF) (KCl 6.8 $mmol.L^{-1}$, KH_2PO_4 0.8 $mmol.L^{-1}$, $NaHCO_3$ 85 $mmol.L^{-1}$, NaCl 38.4 $mmol.L^{-1}$, $MgCl_2.(H_2O)_6$ 0.33 $mmol.L^{-1}$, and HCl 8.4 $mmol.L^{-1}$), $CaCl_2.(H_2O)_2$ (to obtain 0.6 $mmol.L^{-1}$ in the fluid), pancreatin suspension in SIF (based on trypsin activity of 100 $U.mL^{-1}$ in the final mixture), bile solution in SIF (to obtain 10 $mmol.L^{-1}$ in the final mixture), NaOH (volume necessary to adjust the pH to 7.0) and purified water (volume needed to dilute the stock solution of SIF). The samples were incubated for 2 h at 37 °C.

Note that all the electrolyte solutions (SSF, SGF and SIF) were prepared 1.25 × concentrated (i.e. 4 parts of electrolyte stock solution + 1 part water give the correct ionic composition in the simulated digestion fluids) and that $CaCl_2.(H_2O)_2$ was not added to the stock SSF, SGF and SIF solutions to avoid precipitation.

After each phase (oral, gastric and intestinal) of the *in vitro* digestion samples were collected and the gastric and intestinal reactions were stopped by raising pH to 7.0 with $NaHCO_3$ (1 $mol.L^{-1}$) or by adding the enzyme inhibitor pefabloc (1 $mmol.L^{-1}$) (10 µL per 1 mL of sample), for gastric phase sample and intestinal phase sample, respectively.

4.2.4.3 β-carotene bioaccessibility

β-carotene bioaccessibility was determined based on the methodology described elsewhere (Ahmed et al., 2012), assuming that the fraction of the original β-carotene that ended up in the micelle phase was a measure of its bioaccessibility.

Briefly, 10 mL of the microcapsules solution after digestion were centrifuged (18,675x*g*) at room temperature for 30 min. Then, the micelle phase (5 mL) was collected, vortexed with 5 mL of chloroform, and centrifuged at 651x*g*, at room temperature, for 10 min. After centrifugation, the bottom chloroform layer was collected and the extraction procedure was repeated with the top layer.

Pure chloroform was used as reference. The β-carotene concentration extracted from a sample was determined by UV-VIS spectrophotometer (Jasco V560, USA) at 450 nm (absorbance peak). β-carotene concentration was determined from a previously prepared calibration curve of absorbance versus β-carotene concentration in chloroform (0.8 to 10 mg/L).

Bioaccessibility was calculated using the following equation:

$$Bioacessibility(\%) = \left(\frac{C_{micela}}{C_{digestedmaterial}}\right) x100 \qquad \textit{Equation 4-1}$$

Where, C_{micela} and $C_{digestedmaterial}$ are the β-carotene concentrations in the micelle fraction and in the bulk sample (digested material), respectively.

4.2.5 Cell culture

Caco-2 cell line, obtained from human colon carcinoma, was kindly supplied by Department of Biology of University of Minho (Braga, Portugal). All cell culture reagents were purchased from Biochrom GmbH, Biowest, Sigma and Lonza.

Human colon carcinoma Caco-2 cell line was used between passages 50 and 59 (i.e., number of times the culture has been subcultured). Caco-2 cells were grown in culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat

inactivated fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) non-essential aminoacids and 1% (v/v) penicillin-streptomycin, and incubated at 37 °C under 5 % CO_2 water saturated atmosphere. After cells achieving approximately 90 % of confluence, the cells were harvested from flasks with trypsin. The number of viable cells in suspension was estimated by counting in a Neubauer chamber with an inverted microscope (Tibolla et al., 2019).

4.2.5.1 Cell viability assay

The effect of protein and starch microcapsules with β-carotene on cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) conversion assay. The tests were prepared in selected dilutions (10x, 20x, 40x, 80x, 160x of the initial concentration of 10 mL/L) in supplemented DMEM medium and homogenized. The Caco-2 cell suspension was seeded in a 96-well microplate (at 2.5×10^5 cells/mL) in 200 µL of supplemented DMEM and incubated for 24 h at 37 °C in a 5 % CO_2 environment. Then the medium was changed and the test solutions were added to the cell culture and incubated (37 °C; 5 % CO_2) for 24 h. A blank sample (DMEM without cells) and a positive control result (DMEM with cells) were also tested. Each treatment was tested in quadruplicate (Tibolla et al., 2019).

Thereafter, the supernatant was removed and 200 µL of MTT solution (0.5 mg/mL in supplemented DMEM) was added to each well, protected from light and incubated at 37 °C for 4 h to allow purple formazan crystals formation. Then, the medium was removed and formazan purple crystals were solubilized using 200 mL DMSO. The culture plates were shaken on an orbital shaker for 30 min in order to completely solubilize purple formazan crystals. The enzymatic reduction of yellow tetrazolium MTT to a purple formazan was measured by Synergy™ HT Multi-mode Microplate Reader (Biotek Instruments, Winooski, VT, USA) at 570 nm and 690 nm, the latter was used for background subtraction. Cell viability (%) was calculated using Eq. (4-2):

$$Cell\ viability\ (\%) = \frac{A_{exp} - A_{control}}{A_{positive} - A_{control}}\ x\ 100 \qquad \textit{Equation 4-2}$$

where: A_{exp} is the absorbance of the sample (test solution + cells); $A_{control}$ is the absorbance value of the blank sample (DMEM without cells); $A_{positive}$ is the absorbance value of the positive control

(DMEM + cells).

4.2.5.2 Cellular antioxidant activity

Cellular antioxidant activity (CAA) was determined using the method previously described by other authors with some modifications (Silva et al., 2019). In this assay, dichlorofluorescein is trapped in the cells and it is oxidized to fluorescent dichloroffluorescein. This method measures the ability of antioxidants to prevent the formation of fluorescent dichloroffluorescein by AAPH-generated peroxyl radicals (Wolfe and Rui, 2007). Thus, the decrease in the cellular fluorescence when compared to the control cells indicates CAA of β-carotene. Briefly, Caco-2 cells were seeded at a density of 4×10^4 cells/well in a 96-well plate. After reaching confluence (≈ 48 h), cells were washed twice with PBS and triplicate wells were treated and incubated for 1 h with 100 μL of starch and protein amaranth microcapsules with β-carotene 160x concentration dilution and 100 μL of DCFH-DA (25 μmol L^{-1}). Then, the medium was removed, cells were washed with PBS and 100 μL of AAPH (600 μmol L^{-1}) was added in each well. The 96-well microplate was placed into a fluorescence reader (FL800, Bio-Tek Instruments, Winooski, VT, USA) at 37 °C with an emission wavelength of 535 nm and excitation wavelength of 485 nm. The measures were conducted every 5 min for 1 h. Each plate included control and blank wells in triplicate. Control wells contained cells treated with DCFH-DA and oxidant (AAPH) and blank wells contained cells treated with DCFH-DA without oxidant.

CAA of samples was quantified as described by other authors (Wolfe and Rui, 2007). Briefly, after blank and initial fluorescence subtraction, the area under the curve for fluorescence versus time was integrated to calculate the CAA value at each concentration of the sample. The CAA unit was calculated as follows:

$$CAA\ unit = 1 - \frac{AUC_{sample}}{AUC_{control}}\ x\ 100 \qquad \textit{Equation 4-3}$$

The area under the curve (AUC) of fluorescence versus time plot was used to calculate CAA units as described by Wolfe and Rui (2007). CAA units were determined from triplicate determinations of four independent experiments and reported as mean ± standard error.

4.2.6 Data Analysis

Data are expressed as the mean ± SD of independent experiments and were analyzed by one-way analysis of variance (ANOVA) by the statistical analysis of experimental data was performed using IBM SPSS Statistics software (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided *t*-tests (means test of equality) at the 95% significance level ($p < 0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 *In vitro* β-carotene release studies

The β-carotene release from microcapsules was studied during 30 h as can be seen in Figure 4-1.

Regarding 20 % (v/v) ethanol medium, it was observed that both microcapsules were able to release β-carotene, without significant differences ($p < 0.05$) when compared to each other. After 6 h both microcapsules released approximately 80% of the internal β-carotene. Therefore, if the amaranth-based microcapsules are used in alcoholic matrices, they will release most of β-carotene encapsulated.

A similar behavior occurred when the microcapsules were placed in a medium with SDS. Since SDS is a strong cationic detergent, it carries hydrophobic compounds (in this case β-carotene) out of the microcapsule. Morevover, there was significant differences ($p < 0.05$) between starch and protein amaranth microcapsules where starch microcapsules released 91.36 ± 1.52 % of β-carotene and protein microcapsules released 74.9 ± 2.21% of β-carotene, after 30 h. Therefore, the protein microcapsule provided higher protection to β-carotene release. It was difficult to compare the results obtained with data from the literature because similar studies have not been found.

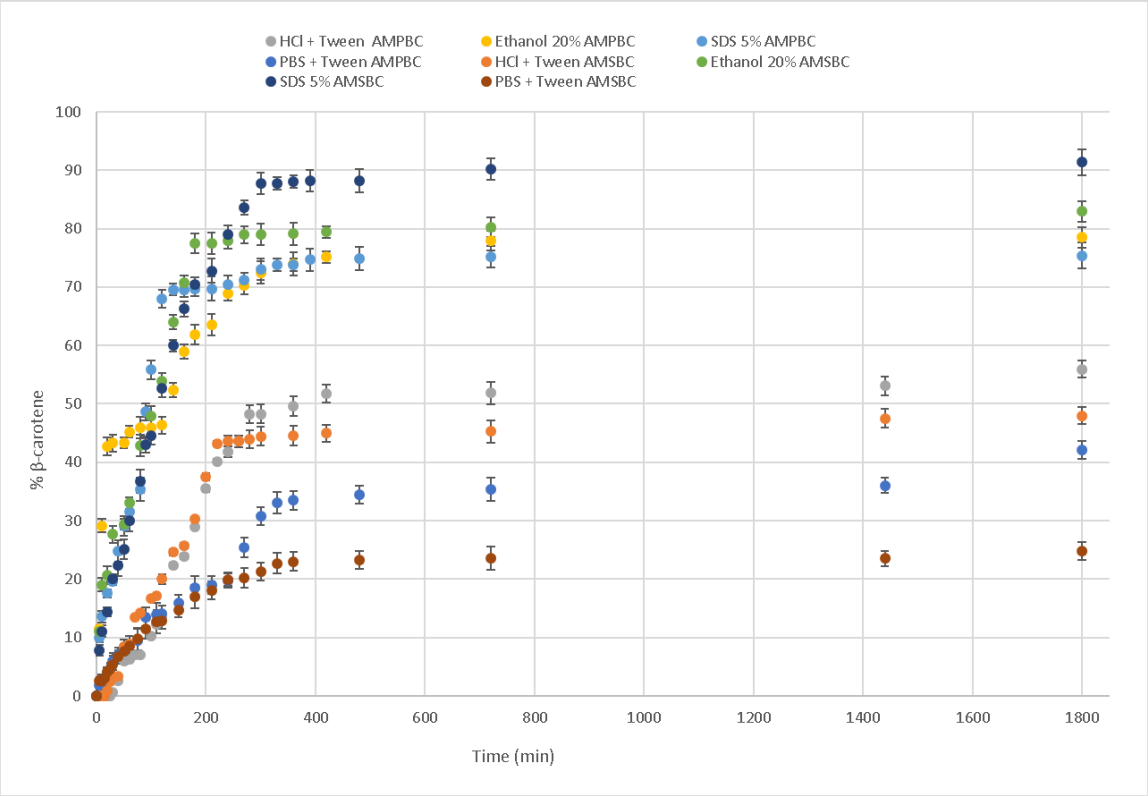

Figure 4-1 - % β-carotene cumulative release in vitro from starch and amaranth protein microcapsules. Mean ± SD (n = 3). AMPBC - Amaranth protein microcapsules with β-carotene. AMSBC – Amaranth starch microcapsules with β-carotene.

The β-carotene release profile from microcapsules in 0.1 mol L^{-1} HCl medium (Fig. 4-1) showed an initial fast release, while after 1 h the release rate became much slower but constant. Probably, the initial release behavior is due to rapid release of β-carotene adsorbed on the surface, or may be associated with loss of wall integrity during particle production or after particle drying (Jain et al., 2016). The slower and sustained β-carotene release after 1 h could be attributed to β-carotene solubilization and diffusion mechanisms (Wang et al., 2010). Therefore, β-carotene trapped in the microcapsules was not completely released in HCl medium during 30 h (reaching a maximum β-carotene release around 45%), showing microcapsules stability during the release test period.

Regarding β-carotene release in PBS medium, there was significant differences between amaranth starch and protein microcapsules ($p < 0.05$), where protein and starch microcapsules released 30.73±1.68% and 24.8±1.73% β-carotene, respectively, during 30 h. Therefore, the

stability of the microcapsules was also maintained at pH 7.4. These results were similar to the results reported by Pinheiro et al. (2012).

The β-carotene release results give an idea of which food products are best suited for incorporating these microcapsules (e.g., mayonnaise, mustard, salad cream, ice cream, beverages), suggesting that they are not suitable to be use in alcoholic beverages.

4.3.2 Bioaccessibility

Free β-carotene and starch and protein microcapsules incorporating β-carotene were subjected to *in vitro* digestion analyze to study the impact of microencapsulation on β-carotene bioaccessibility.

β-carotene bioaccessibility in microcapsules was 22.1 ± 2.1 % and 33.8 ± 3.1 % for amaranth starch microcapsules with β-carotene and amaranth protein microcapsules with β-carotene, respectively, being statistically different ($p < 0.05$). These results corroborate the bioaccessibility in β -carotene results presented in other works: 26 ± 1.5% (Gómez-Mascaraque et al., 2017); 13.8 ± 1.1% (Liu et al., 2018); 17.45 ± 1.99% (Roman et al., 2012a) and 20% (Iddir et al., 2019).

A determining factor that affects the absorption of β-carotene is the presence of proteins, as seen in our study. Caseins have been successfully used for β-carotene microencapsulation, significantly improving their bioaccessibility (Soukoulis and Bohn, 2018; Yi et al., 2014). After their adsorption to the lipid surface of the droplets, proteins can stabilize oil-in-water emulsions in the GI tract, attributed to the fact that proteins can be highly active molecules on the surface (Soukoulis and Bohn, 2018), and the particles formed tend to be highly negative, preventing the aggregation of lipid droplets (Qiu, et al., 2015), in this study we had corn oil as a carrier.

The bioaccessibility of β-carotene is around 28 % in mixed diets (normal omnivorous diets) and 53 % in diets rich in oil (Van Loo-Bouwman et al., 2014). For example, the bioaccessibility of β-carotene is 12 to 18 % for a meal of carrots, spinach and chicken, 22 to 16% for a mango and chicken meal, all mixed diets (Garrett, Failla and Sarama, 1999).

Several studies have shown that dietary fibers coating can be used to control the biological fate of delivery systems during digestion. For example, Amyoony (2014) investigated guar gum in

the biological destination of stabilized β-carotene emulsions. It was found that the high viscosity formed by guar gum would capture micelles or bile salts and delay diffusion, leading to decreased diffusion of lipolysis and bioaccessibility of β-carotene. In addition, not only properties such as viscosity and structure affect the digestion properties in GIT, but also the physico-chemical characteristics of fibers. The effect of the extension of the methyl esterification of pectin on the bioaccessibility of β-carotene in emulsions was studied by Verrijssen et al. (2014). The authors showed that less β-carotene bioaccessibility was achieved in emulsions stabilized by lower pectin esterified with methyl. Finally, the bioaccessibility of β-carotene was lower in the emulsion that contained low pectin esterification, which can be attributed to the lower degree of lipolysis in the emulsion with larger droplet size and greater viscosity of the digesta. Dietary fibers influence lipolysis and reduce the bioaccessibility of β-carotene in lipid-based microcapsules, corroborating the analysis of starch and protein extracted from amaranth, where starch had 2.6 g of fiber/ 100 g product and the protein had 3 times less (Chapter 3; subchapter 3.3.1 of this thesis).

β-carotene was not detected on free β-carotene mixed miceles, therefore bioaccessibility was considered insignificant. Probably, the crystalline β-carotene and its insoluble free form degraded in the stomach and therefore did not reach the micelles. Do et al. (2014) reported that low triolein and canola oil amounts (0, 5 and 1 %) were required to achieve maximum carotenoid mycelization, while higher oil amounts were required (~2.5 %) when the oily triglycerides were mainly medium chain saturated fatty acids (Do et al., 2014). In the current thesis study, β-carotene was only detected in the microencapsulated β-carotene mixed micelles.

Ideally, β-carotene release should occur in the intestine where absorption occurs. However, the low pH of the gastric phase may increase the cis β-carotene isomer content, which has been previously described for other carotenoids (Gómez-Mascaraque et al., 2017). pH isomerization is considered at least partially responsible for the high proportion of carotenoid cis isomers found in the human body after consumption (Moraru and Lee, 2005; Re et al., 2001).

These results showed that β-carotene microencapsulation within amaranth protein and starch matrices produced by spray-drying were a good approach to improve β-carotene bioaccessibility. Emulsification had already been described as an effective approach to increase bioaccessibility of β-carotene (Qian et al., 2012), but encapsulation techniques and spray-drying add the advantage of obtaining an easy-to-handle dry powder as an ingredient.

The fatty acid saturation degree impacts the carotenoids incorporation in mixed micelles and, consequently, its bioaccessibility and bioavailability (Gómez-Mascaraque et al., 2017). For example, carotenoids showed greater extent of micellarization in the presence of triolein and canola oil than in trioctanoin and coconut oil (Dong et al., 2013). The main fatty acid in canola oil and triolein is oleic acid, which represents only 25-30 % of total fatty acids in soybean oils (Rodas and Bressani, 2009b). Given the decisive impact that the carrier oil lipid profile has on carotenoid bioaccessibility, further work should study the impact of different carrier oils on β-carotene microencapsulation and their subsequent bioaccessibility in order to improve the potential of this technique.

4.3.3 Cell viability assay

It is important to evaluate the cytotoxicity of the polymeric matrices of the microcapsules since the presence of toxic substances can cause damage to cells and the loss of absorption of bioactive compounds (Sedaghat Doost et al., 2019). In order to evaluate any potential protein and starch microcapsules with β-carotene cytotoxicity, Caco-2 cells were exposed to different β-carotene concentrations for 24 h. The effects of protein and starch microcapsules with β-carotene on Caco-2 cell viability are shown in Fig. 4-2.

In general, Caco-2 cells viability slightly decreased as β-carotene concentration increased (Figure 4-2). Regarding free β-carotene, amaranth starch and protein microcapsules and amaranth starch microcapsules incorporating β-carotene, more than 80 % of the cells remained viable when exposed to 10x β-carotene dilution. Only the amaranth protein microcapsules incorporating β-carotene had higher cytotoxicity at 10x dilution. However, theses microcapsules showed low cytotoxicity at 80x dilution, where more than 90 % of cells remained viable. And from this dilution, there are no longer any significant differences when compared to the control.

Comparing to the control group (i.e., 100 % cell viability), the cell viability was not significantly affected (p>0.05) after exposure to 80x and 160x dilution for all samples. However, cells exposed to the protein microcapsules with β-carotene samples at 10x, 20x and 40x significantly reduced (p<0.05) cell viability to 67.49 %, 64.54 % and 68.82 %, respectively, when compared to the positive control group. Our study showed that starch and protein microcapsules

with a maximum of 160x β-carotene dilution can be used without compromising cell viability. Therefore, 160x dilution concentration was used in the following cell studies.

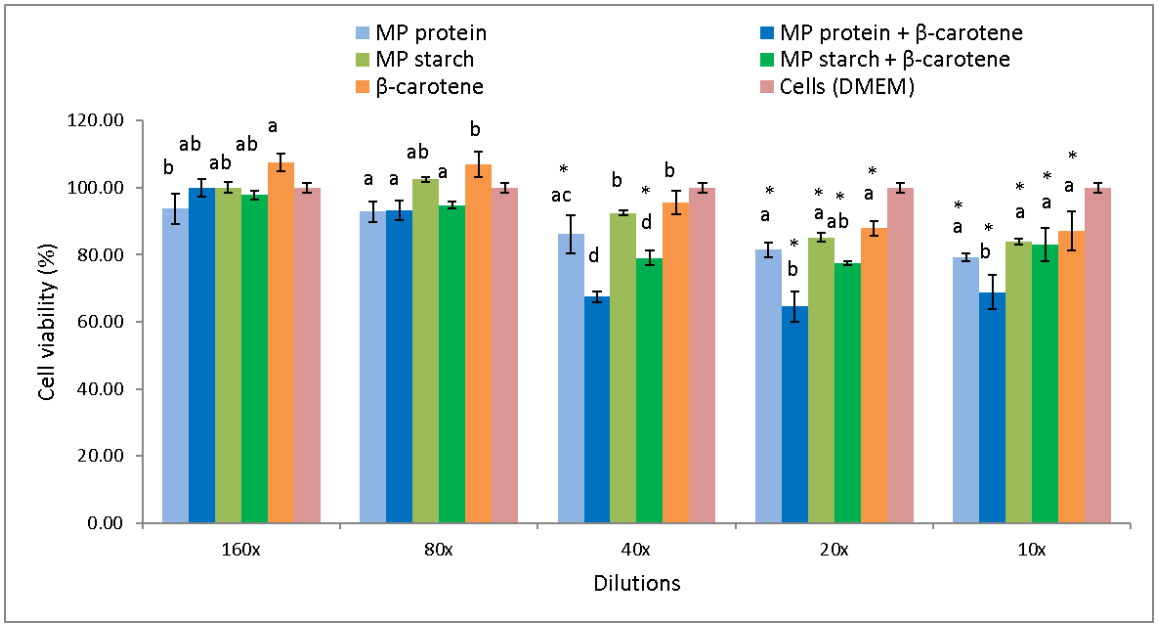

Figure 4-2 - Cell viability of Caco-2 cells after incubation with starch and protein amaranth microcapsules (MP), free β-carotene and starch and protein microcapsules with β-carotene (bars represent standard deviation). a,b,c,d Values with different letter superscripts for the same concentration were statistically different (p≤0.05). The asterisk (*) indicates significant difference (p ≤ 0.05) relative to the control.

Previous *in vitro* studies showed that oil-in-water microemulsions with 0.03125% (v/v) β-carotene were not cytotoxic to Caco-2 cells, and viability was higher than 90% (Roohinejad et al., 2015). Han et al. (2019) evaluated the micelles of stabilized β-carotene emulsions and also found non-toxic results.

4.3.4 Cellular Antioxidant Activity of β-Carotene Microcapsules

Popular antioxidant activity/capacity assays (such as ORAC, TRAP, TEAC, TOSC, PSC, and FRAP) show the inability to represent the complexity of biological systems. They measure chemical

reactions only, and these reactions cannot be interpreted to represent activity *in vivo*, as they cannot account for the bioavailability, stability, tissue retention, or reactivity of the compounds under physiological conditions (Wolfe and Rui, 2007). *In vitro* chemical assays have advantages for the initial screening of different formulations because they are relatively simple, rapid, and low cost to perform. However, they do not accurately reflect the potential antioxidant activity of formulations *in vivo* (Chen, Li, Li, McClements, and Xiao, 2017). In future studies, it will be important to determine the antioxidant activity of the samples after oral administration to animals or humans. However, these types of studies are more expensive, time-consuming, and ethically challenging than simple cell culture models, and should therefore only be carried out once the safety and potential efficacy of a new ingredient or delivery system have been established.

Dichlorofluoroscin diacetate (DCFH-DA) is easily absorbed into cells where esterases cleave the acetate groups producing the more polar water soluble dichlorfluoroscin (DCFH) that is retained by the cell. Addition of ABAP, a peroxyl radical source, results in the production of the fluorescent 2',7'-dichlorofluorescein (DCF). In order to quench the peroxyl radical oxidation the antioxidant must also be present in the cell.

In the present study, DCFH-DA was used as a fluorescent probe to evaluate the CAA of different samples at 160x (125 μg/L) β-carotene dilution. Caco-2 cells were used as a model to analyze the antioxidant response to free and encapsulated β-carotene. As shown in Figure 4-3, the microcapsules encapsulating β-carotene exhibited ability to inhibit DCFH oxidation induced by peroxyl radicals.

All samples CAA values were statistically different when compared to each other ($p < 0.05$). Based on CAA results, β-carotene encapsulated in amaranth starch improved the β-carotene antioxidant activity when compared to amaranth protein microcapsules. Both free and encapsulated β-carotene quench ABAP produced by peroxyl radical reaction showing that β-carotene is incorporated into Caco-2 cells. The CAA values of all microcapsules with β-carotene encapsulated were significantly higher ($p < 0.05$) compared to free β-carotene. Similar studies showed that encapsulation of β-carotene improved CAA values (Gu et al., 2018; Yi et al., 2015; Du et al., 2019). This may be due to higher surface area of the small capsules and an amorphous solid state in microcapsules compared to crystalline free β-carotene.

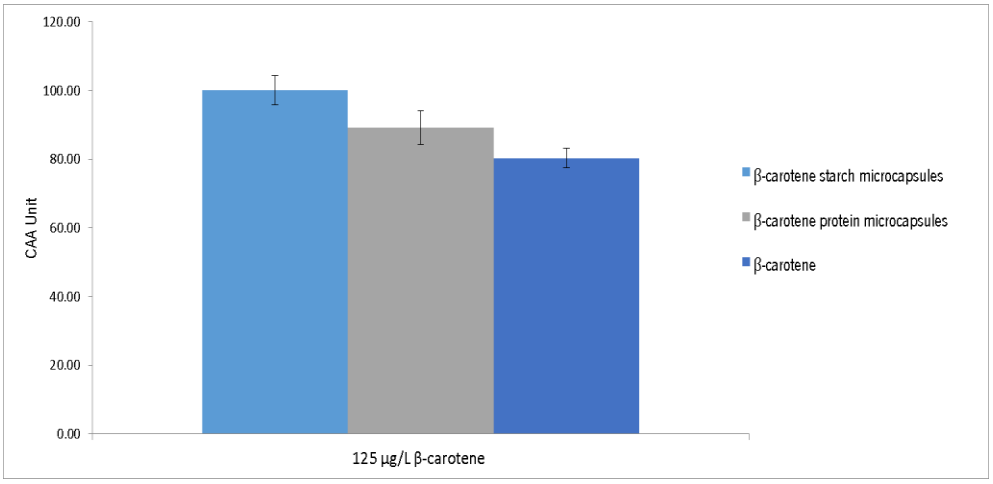

Figure 4-3. Cellular antioxidant activity of samples at 125 µg/L β-carotene on Caco-2 cells. Values reported are mean ± standard deviation (SD).

4.4 CONCLUSIONS

The purpose of this study was to investigate the effect of the amaranth protein and starch microcapsules with β-carotene on its release in different food simulants, β-carotene bioaccessibility, CAA and cytotoxicity using Caco-2 cells.

β-carotene stability in various *in vitro* release food media was demonstrated in acid and PBS media, but not on alcoholic media within 30 h.

The results showed that β-carotene microencapsulation in the protein and amaranth starch matrices by spray drying was a good approach to improve the bioaccessibility of β-carotene, with a more significant result when using the protein extracted from the grain.

It has also been shown that starch and protein microcapsules were not cytotoxic at 125 µg/L (160x) β-carotene.

In the present study, the CAA values of the microcapsules with β-carotene were around 89% and 100% for starch and protein microcapsules, respectively, indicating that the microcapsules exhibited the ability to protect β-carotene and to enhance its antioxidant performance.

This work contributed to a better understanding of how these microcapsules behave within the human GI tract and this knowledge will be useful for the optimization of delivery systems that improve the physicochemical stability of β-carotene in foods.

This study suggests that the developed microcapsules can be used as platforms for the design of functional foods with different functionalities such as increased β-carotene bioavailability.

Further studies are needed to evaluate microcapsules performance once incorporated in food matrices followed by digestion/absorption simulation. Moreover, *in vivo* studies are needed to determine their functionality under realistic GI conditions.

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SECTION III

AMARANTH AS A SOURCE OF BIOACTIVE COMPOUNDS TO BE IMMOBILIZED IN EDIBLE FILMS

CHAPTER 5

DEVELOPMENT AND CHARACTERIZATION OF ALGINATE FILMS WITH PHENOLIC COMPOUNDS OBTAINED FROM AMARANTH GRAIN

This chapter explores the preparation of alginate-based food packaging materials with the incorporation of valuable phenolic compounds extracted from amaranth. The films were characterized in terms of mechanical (tensile strength and elongation at break) and thermal (DSC and TGA) properties, opacity, water sensitivity (moisture content, solubility and water vapor permeability) and chemical and conformational analyzes with FTIR.

The results presented in this Chapter were adapted from:

Coelho, L.M., Faria, C., Madalena, D., Martins, J.T., Pinheiro, A.C., Genisheva, Z., Vicente, A.A., Development and characterization of alginate films with phenolic compounds obtained from amaranth grain. *Journal of Polymers and the Environment* (submitted).

5.1 INTRODUCTION

Plastic films are composed mainly of polypropylene or polyvinyl chloride and do not degrade easily (Liling et al., 2016). The accumulation of these plastic films in the soil reduces soil permeability and fertility, and consequently reduces crop yields (Aadil, Prajapati, and Jha, 2016). Lipids, proteins and polysaccharides are natural macromolecules and biodegradable materials that can be used as an alternative to the plastic films. Alginate is a polysaccharide extracted mainly from brown algae cell walls and is composed of mannuronic acid and guluronic acid (Kim, Baek, and Song, 2018). Alginate is renewable and non-toxic, and has excellent film-forming properties.

Natural antioxidants can be added into packaging materials to enhance their functionality by conferring antioxidant properties (Dou et al., 2018). Recently, natural antioxidants have received attention as replacement for synthetic preservatives in the food industry. Antioxidants play a vital role in free radical elimination and chain breaking of oxidation reactions *in vivo* and *in vitro*. Potential uses of antioxidants include the prevention of diseases related to oxidative stress in humans (e.g., pharmaceuticals and cosmetics) as well as reactions in food products {Formatting Citation}. The use of synthetic antioxidants such as propyl gallate, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food causes many side effects, being its use constantly under review by the FDA (Basanta et al., 2018). Consequently, there is a growing interest in finding natural antioxidants substances capable of eliminating free radicals and hindering oxidation rancidity, delaying food spoilage (Zhang, Yang, and Liu, 2009). Phenolic compounds are secondary metabolites of plants that have a phenol ring (phenolic acids, phenolic alcohols) or various ring aromatic compounds with one or more hydroxyl groups (i.e., polyphenols) (Borges et al., 2017). *In vivo* and *in vitro* experiments have revealed that most phenolic compounds, especially polyphenols, have many different bioactivities such as antioxidation, digestive enzyme inhibition and anti-inflammation (Huang et al., 2017). The application of phenolic compounds as functional ingredients is widely studied. In a complex food system, interactions between these bioactive compounds and the food matrix may affect the quality of food rich in polyphenol products, as these interactions affect both bioavailability and bioactivity of phenolic compounds (Gómez-Mascaraque et al., 2017).

Within the relatively recent concept of active packaging, the incorporation of antioxidants or antimicrobials in packaging materials is useful for extending shelf life and improving food safety or sensory properties (Valdés et al., 2014). Most developments aim at directly incorporating active

compounds into the polymer matrix of the packaging, while maintaining or improving the barrier and mechanical properties of the bulk material. Currently, natural compounds with antioxidant properties have produced significant interest and can potentially be used in food packaging to replace synthetic antioxidants as they may be biologically degradable and are commonly considered safe migrants (Dainelli et al., 2008). For example, the addition of antioxidants such as α-tocopherol or citric acid to edible starch-chitosan mixtures resulted in good antioxidant capacity of the films but also good barrier properties (Bonilla et al., 2013).

Amaranth is a pseudocereal with great potential to prevent malnutrition especially in the low-income food-deficient countries. Additionally, amaranth grains are gluten-free and a rich source of bioactive compounds with antioxidant properties. These non-saponiferous substances from the lipid fraction of grains that include squalene, tocopherols, sterols, and others, have antithrombotic, antioxidant, hypocholesterolemic, antidiarrheal, antidepressant, and anticancer effects (Coelho et al., 2018). Amaranth grains contain phenolic compounds (e.g., flavonoids) and a relatively high antioxidant capacity (Barba de la Rosa et al., 2009) where total phenolic acids in several amaranth ecotypes ranged from 16.8 to 40.1 mg/100 g (dry weight) (Repo-Carrasco-Valencia et al., 2010). However, the phytochemical composition depends on multiple variables during growing conditions.

Amaranth flour films have already been described (Condés et al., 2018; Sobral, Menegalli, and Tapia-blacido, 2005; Tapia-blácido, Amaral, and Menegalli, 2011). However, the extraction of phenolic compounds from amaranth grain and their incorporation on alginate-based films has not yet been performed. Therefore, this research work could provide an efficient alternative for the food industry, since amaranth biological activities could enhance the edible films' properties. Furthermore, phenolics incorporated in a film may be a pathway to increase phenolic stability, antioxidant efficiency and food shelf life. In this context, in this study alginate films with phenolic compounds extracted from amaranth grains have been developed and characterized.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Amaranth (*Amaranthus cruentus*) grain was originated from Peru and it was stored in a cold chamber until further analysis. Glycerol, alginate, ethanol, methanol gallic acid, caffeic acid,

pyrogallol, ferulic acid, chlorogenic acid, 2,2-Diphenyl -1-picrylhydrazine (DPPH) and Folin-Ciocalteu reagent (2 mol L^{-1}) were purchased from Sigma-Aldrich (Saint Louis, USA).

5.2.2 Methods

5.2.2.1 Amaranth phenolic extraction

Amaranth grains were sieved, cleaned and ground in a roller mill to obtain whole amaranth flour. Amaranth flour was stirred for 1 h at room temperature, using different extraction solvents - ethanol (80 %), methanol (50 %) and water – at a 1:20 (amaranth:solvent) ratio (Wang et al., 2010). Subsequently, the organic solvents were evaporated at 35 °C under vacuum and the residual extract was freeze-dried (CHRIST, ALPHA 1-4 LD PLUS). The dry residue weight was determined gravimetrically and stored at -20 °C until further use.

5.2.2.2 Identification of amaranth phenolic compounds by ultra-high-performance liquid chromatography (UHPLC)

The amaranth phenolic extracts were homogenized and analyzed using UHPLC. Samples were analyzed by the Shimatzu Nexpera X2 UHPLC Diode Array Detector (Shimadzu, SPD-M20A) chromatographic program.

Separation was performed on a reverse phase Aquity UHPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm particle size from Waters) and a pre-column of the same column material at 40°C. The flow rate was 0.4 mL/min.

The HPLC grade solvents used were formic acid (0.1 %) as solvent A and acetonitrile as solvent B. The elution gradient for solvent B was as follows: 0.0 to 5.5 min eluent B to 5%; 5.5 to 17 min a linear increase to 60%; 17.0 to 18.5 min, a linear increase to 100 %, then column balance from 18.5 to 30.0 min to 5%. Phenolic compounds were identified by comparing their chromatograms and retention times with the corresponding standards. The compounds were quantified and identified at different wavelengths. All analyzes were done in triplicate.

5.2.2.4 Antioxidant activity of freeze-dried amaranth extracts

Approximately, 1 g of lyophilized amaranth extract was weighed and 50 mL distilled water was added to the extract. The freeze-dried extract was suspended in the water using a roto-stator for about 1 min and afterwards stirred for about 12 h at 200 rpm at room temperature.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined using the methanolic extract according to the method reported by Omodamiro et al., (2016) (Omodamiro, Jimoh, and Ezurike, 2016). A DPPH stock solution (24 mg DPPH/100 mL methanol) was prepared and stored at -20 °C. From this stock solution the working solution (8.6 mL stock solution/50 mL methanol) was prepared. The reaction was performed with 150 μL of the extract and 2.9 mL of the working solution. After 30 min of reaction in the dark, the sample was analyzed in a spectrophotometer at 515 nm. The calibration curve was prepared with the water-soluble ascorbic acid at concentrations from 25 to 935 μmol L^{-1}. Results were expressed as mg ascorbic acid equivalent/g sample.

5.2.2.5 Total phenolic content of freeze-dried amaranth extracts

The extracts total phenolic content was determined using the Folin-Ciocalteau reagent as described by Gamel et al. (2005). Lyophilized amaranth extract (1 g) was added to 2 mL of aqueous Na_2CO_3 solution (2 %) in glass tubes. After 2 min, 100 mL of Folin-Ciocalteau reagent diluted with distilled water (1:1) was added to the mixture and vortexed. The solution was allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm against reaction blank using a spectrophotometer (Beckman Coulter, DU 640, Beckman Instruments Inc., Fullerton, CA, USA). The blank was prepared by replacing the sample with water in the reaction. Results were expressed as gallic acid equivalents (mg GAE/100 g dry sample) using standard gallic acid curve dissolved in distilled water at 0.02 to 0.12 mg/mL.

5.2.2.6 Production of alginate film with amaranth bioactive compounds

To understand the effects of alginate, glycerol and amaranth phenolic compounds on films properties, a complete factorial design was elaborated, with three central points and four axial points and a total of 9 trials. The variables studied were alginate (0.5 to 1.5 % v/v), glycerol (0 to 1.0 % v/v) and phenolic compounds (0 to 1.5 % v/v) based on a previous study (Perales-Sánchez et al., 2014), as described in Table 5-1.

Table 5–1 - Experimental design of films' composition (i.e. different alginate and glycerol concentrations (at a fixed final concentration of 1.5 % in the mixture), and different amaranth phenolic compounds concentrations.

Sample	Alginate (%)	Glycerol (%)	Phenolic compounds (%)
1	1.15	0.35	0.50
2	0.75	0.75	0.50
3	1.15	0.35	1
4	0.75	0.75	1
5	1.5	0	0.75
6	0.5	1.0	0.75
7	1.0	0.5	0
8	1.0	0.5	0.75
9	1.0	0.5	1.50

Alginate film-forming solutions were prepared by dissolving alginate in stirring distilled water (350 rpm) at room temperature for 30 min. Then, glycerol was added and solutions were stirred at 350 rpm for 30 min at room temperature until a homogeneous solution was obtained (Botelho et al., 2015).

The freeze-dried phenolic compounds were added to the solutions according to the concentrations defined in Table 5.1. The solutions were heated and maintained at 40 °C for 1 h at 900 rpm. Then, 28 mL aliquots were transferred to acrylic plates, and the plates were dried in a

circulating oven at 35 °C for 20 h. After drying, the films were stored at room temperature (20 ± 2 °C), in desiccators containing a saturated solution of $Mg(NO_3)_2.6H_2O$ at 53 % of relative humidity (RH) until further analysis.

5.2.2.7 Characterization of the films

5.2.2.7.1 Water contact angle

The water contact angle was measured by the sessile drop method (Kwok and Neumann, 1999). A water droplet (5 µL) was deposited on the film surface with an automatic piston syringe (Hamilton, Switzerland). The drop image was photographed using a digital camera. An image analyzer was used to measure the angle formed between the surface of the film in contact with the drop, and the tangent to the drop of liquid at the point of contact with the film surface. The measurements were performed within 180 s after dropping coating solution onto film surfaces, all made in triplicate.

5.2.2.7.2 Film Solubility

The solubility of the films in water was determined according to the method reported by other authors Rezende, Nogueira, and Narain (2018), being defined by the solubilized dry matter content after 24 h of immersion in water.

The initial dry matter content of each film was determined by drying it until constant weight in an oven at 105 °C. Two film discs (2 cm in diameter) were cut, weighed and immersed in 50 mL of water. After 24 h of immersion at 20 °C (with occasional agitation), the film pieces were removed and dried to constant weight in an oven at 105 °C in order to determine the weight of dry matter that has not been solubilized in water.

5.2.2.7.3 Moisture content

The moisture content (MC) was expressed as the percentage of water removed from the initial mass sample. MC was determined gravimetrically by drying film samples (of about 20 mg) at 105 °C in an oven with forced air circulation for 24 h. The experiments were performed on each film sample in triplicate.

5.2.2.7.4 Water vapor permeability (WVP)

WVP was determined gravimetrically using the ASTM E96-92 method described by Casariego et al. (2009). Three samples (diameter of 5.1 cm) were cut from each film. Each sample was sealed on a permeation cell (i.e., cup containing distilled water at 100 % RH; 2.337×10^3 Pa vapor pressure at 20 °C) and placed in a desiccator containing silica gel (0 % RH; 20 °C).

The water transferred through the test films was determined from cup weight loss over time. The cups were weighed to the nearest 0.1 mg at 2 h intervals. The steady state of weight loss was reached after 10 h. WVP [(g/(m.s.Pa)] was calculated using the following equation:

$$WVP = \frac{(WVTR \times L)}{\Delta P}$$

Equation 5-1

Where WVTR is the water vapor transmission rate measured through the film (g m^{-1} s^{-1}) (calculated from the slope of the curve divided by the area of the film = 15.20 cm^2), L is the average film thickness (m) and ΔP is the partial pressure difference of water vapor (Pa) throughout the film (Cerqueira et al., 2011).

For each type of film, WVP measurements were replicated three times and the coefficient of variation obtained was below 5 %.

5.2.2.7.5 Thermal analysis

The thermal stability and degradation profile of all films were evaluated by thermogravimetric analysis (TGA) with a Shimadzu TGA 50 according to Souza et al., (2009).

Film samples (approximately 5 mg) were placed under high pressure in stainless steel pans. An empty pan of the same type used to the samples was used as a reference. The samples were heated at a constant rate of 10 °C min, from 20 to 580 °C, under nitrogen atmosphere, in triplicate.

Differential scanning calorimetry (DSC) measurements were performed with a Shimadzu DSC 50 (Shimadzu Corporation, Kyoto, Japan). A film sample (10 mg) was placed in aluminum pans and measurements were taken between 20 and 250 °C at a heater rate of 10 °C under a nitrogen atmosphere. Data were analyzed using TASYS software (Shimadzu Corporation, Kyoto, Japan), in triplicate. Melting enthalpy change (ΔH_m) and melting temperature peak (T_m) were determined.

5.2.2.7.6 Mechanical properties

Tensile strength (TS), elongation at break (EB) and the Young modulus (YM) of the film samples were measured with a texturometer (Texture analyzer TA.XT2i) according to ASTM standard method (ASTM D 638 - 99, 2000). The samples (45x20 mm^2 strips) were fixed between the grips with an initial separation of 100 mm, and the cross-head speed was set at 0.8 mm s^{-1}. At least five measures were made for each film (Martins et al., 2015).

The TS was calculated by dividing the maximum load (N) by the initial film cross-sectional area (m^2) of the film, and was expressed in MPa. EB is a measure of the flexibility of the film. EB was expressed as a percentage and was determined as the ratio of the final length of the specimen at the point of break to the initial length of the specimen.

5.2.2.7.7 Film thickness

Film thickness was measured with a digital micrometer device (No. 293-5, Mitutoyo, Japan) with a resolution of 0.001 mm. Ten thickness measurements were taken on each film sample at different randomly chosen points and the mean values were used for water vapor permeability calculations.

5.2.2.7.8 Optical properties

Film color was determined with a Minolta colorimeter (CR 300; Minolta, Japan). A white plate (Y = 93.5, x = 0.3114, y =0.3190) was used as standard for calibration. The brightness (L^*) and the chromaticity parameters a^* (red - green) and b^* (yellow - blue) were measured.

Measurements were made by placing the film sample over the standard. Samples were analyzed in triplicate, recording four measurements for each sample.

The opacity of a material is an indication of how much light passes through the film. The higher the opacity, less light passes through the material. Generally, opacity is calculated from reflectance measurements. The opacity of the samples was determined according to the Hunterlab method, as the relationship between the opacity of each sample on a black standard and the opacity of each sample on a white standard. Six measurements were taken of each sample, and three samples of each film were tested.

5.2.2.7.9 Fourier Transform IR (ATR-FTIR) spectroscopy

For this analysis, ATR-FTIR spectrophotometer with attenuated total-infrared reflectance spectroscopy (ATR-MIR) with resolution of 4 cm^{-1} was used, in the range of wavelengths between 650 cm^{-1} and 4000 cm^{-1}, and 128 scans were performed. The spectra of each sample were obtained after placing the sample on the ATR-MIR cell crystal and five readings for each sample were performed (Pinheiro et al., 2012). The spectra were initially obtained in reflectance mode and the characteristic groups of each band were identified.

5.2.2.8 Scanning electron microscopy (SEM)

The surface morphology of the films was analyzed by high resolution, using a desktop scanning electron microscope (SEM) (Phenom-World BV, Netherlands)). All results were acquired using the ProSuite software.

The samples 1, 5, 7 and 9 were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 2 nm of Au (20 Å) for improved conductivity. The aluminium pin stub was then placed inside a Phenom Standard Sample Holder, and analysed with surface topography at 5kV. Various areas of the film surface (10 mm/ 10 mm) were checked using touch mode.

5.2.2.9 Statistical analyses

Statistical analyses were performed with Microsoft Windows Excel 2019 and software IBM SPSS Statistics Base 22.0. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed to determine the significance of differences with α=0.05.

The principal component analysis (PCA), the k-means cluster analysis, the box plot (for outlier detection) and Pearson correlation matrix, with a 2-tailed test of significance with α=0.05 to obtain the significance level of the correlation coefficients, were obtained using Origin Pro 2018 (OriginLab Corporation). It is important to refer that the sample 6 was not analyzed since it was not possible to handle the film sample and to perform the characterization tests.

5.3 RESULTS AND DISCUSSION

5.3.1 Antioxidant activity and total phenolic content of amaranth extracts

The antioxidant ability of the samples is shown in Table 5-2.

Table 5-2 - Total phenolic content and antioxidant activity (AA) of Amarathus cruentus sp. extracts in different solvents and after freeze-drying.

Solvent	TPC (mg GAE/g)	AA (mg ACE/g)
Methanol (50%) [1]	3.9 ± 0.12[a]	0.24 ± 0.01[a]
Ethanol (80 %) [1]	3.5 ± 0.09[a]	0.21 ± 0.01[b]
Water	2.4 ± 0.07[b]	0.12 ± 0.02[b]
Freeze-dried ethanol extract	16.78 ± 2.61[c]	7.54 ± 0.29[c]

GAE gallic acid equivalent; ACE ascorbic acid equivalent; AA antioxidant activity; TPC total phenolic content.

[1] Expressed as a solvent fraction in water.

[a-b-c] Different letters in the same column indicate statistical differences between samples (p<0.05).

All analyzed amaranth extracts were able to react with free radicals and convert them into stable products. The total phenolic content in the methanolic extract (3.9 mg GAE/g sample) and ethanolic extract (3.5 mg GAE/g sample) was significantly higher than in aqueous extracts (2.4 mg GAE/g sample) (Table 5-2). These results can be explained by the interaction of antioxidant compounds and/or the solubility of the compounds with the solvent used. On the other hand, methanol and ethanol extracts mainly contain hydrophobic compounds, such as phenolic and amino acids and hydrophobic peptides (Karamać et al., 2019). It is important to note that Folin-Ciocalteau is not specific for phenolic compounds and can be reduced by other compounds such as tertiary aliphatic amines, ascorbic acid, Cu (I), sugars, aromatic amino acids (tryptophan, phenylalanine and tyrosine) among others (Huang, Boxin and Prior, 2005; Michalska, Ceglińska and Zieliński, 2007). Pazinatto et al., (2013) observed similar extraction behavior for amaranth products. Solvents with different polarities cause differences not only in the extracted phenolic compounds, but also in the antioxidant capacity found in the extracts (Gorinstein et al., 2007).

An increase in total phenolics was revealed after lyophilization of the ethanol extract, because the extract was concentrated through drying.

5.3.2 Identification of amaranth phenolic compounds by UHPLC

The ethanol extract was chosen to perform the analysis of phenolic compounds, since ethanol is a solvent approved by the FDA for use in food and there was no significant differences ($p>0.05$) between ethanol and methanol extracts (Table 5-2).

The results obtained regarding phenolic compounds identified in the mixture extracted from amaranth are presented in Table 5-3.

The principal antioxidant compounds detected by HPLC in ethanol extracts of *A. cruentus* were vanillic acid acid and resveratrol (Table 5-3). These results are in accordance with the ones reported by other authors (Conforti et al., 2005). Moreover, Pedersen et al. (2010) found three polyphenols (rutin, isoquercitin and resveratrol), and rutin present at higher concentration). The other amaranth compounds had similar concentration values to our results.

Table 5-3- Phenolic acids and flavonoids present in the freeze-dried amaranth (Amarathus cruentus sp.) grain extract

Phenolic compounds	Concentration (mg/L)
Vanillic acid	6.08 ± 0.01
Ferulic acid	1.96 ± 0.02
p-coumaric acid + epicatechin	1.66 ± 0.00
o-coumaric acid	1.32 ± 0.00
Ellagic acid	1.08 ± 0.13
Cinnamic acid	2.23 ± 0.01
Resveratrol	4.07 ± 0.02
Rutin	2.42 ± 0.00
Total	20.82 ± 0.10

According to other authors, the major polyphenolic compounds identified in *Amaranthus cruentus* was ferulic acid (120–620 mg/kg), whereas vanillic acid (15.5–69.5 mg/kg), benzoic acids (4.7–136 mg/kg), caffeic acid (6.41–6.61 mg/kg) and p-coumaric acid (1.2–17.4 mg/kg) were detected at minor concentrations (Repo-Carrasco-Valencia et al., 2010). Additionally to these

phenolic acids, some monomeric and dimeric flavonoids has been detected in *Amaranthus cruentus* seed samples as quercetin (214–843 mg/kg), kaempferol (22.4–59.7 mg/kg), isorhamnetin (42–600 mg/kg), rutin (7–592 mg/kg), among other minority flavonoids (Venskutonis and Kraujalis, 2013).

Rutin (quercetin-3-O-rutinoside) and quercetin (the precursor of rutin) are flavonoids ubiquitously found in nature and in amaranth which may be useful for the prevention and treatment of different types of cancer (Coelho et al., 2018). Both rutin and quercetin, present also important antioxidant properties and produce a significantly inhibition of the oxidation of high-density lipoprotein (HDL) cholesterol (Kalinova and Dadakova, 2009).

5.3.3 Solubility, moisture content, water vapour permeability (WVP) and water angle contact measurements of films

Water sensitivity is one of the major problems of polysaccharide-based films, and is evaluated by different methods such as monitoring moisture content, water activity and sorption, solubility, contact angles and through WVP measurement (Martins et al., 2012).

Surface tension characterization is one of the key properties of packaging materials, being performed by measuring the contact angle between the film surface and water, acting as an indicator of the film surface hydrophobicity (Jridi et al., 2019a).

Table 5-4 shows the water contact angles of alginate films with amaranth phenolic compounds. Comparing samples 1 and 2, which have the same amount of phenolic compounds, it is noticeable that sample 1 is more hydrophilic since its contact angle is lower than that presented for sample 2. This situation is also reflected in samples 3 and 4. For the same reason presented above, it can be concluded that films with higher alginate concentration than glycerol, have lower hydrophobicity. Comparing film 9 to sample 7, which does not contain phenolic compounds, it can be concluded that sample 7 film surface has a more hydrophobic character since the increase of the contact angle amplitude is directly proportional to the increase in hydrophobicity of the film surface (Rotta et al., 2009).

Table 5-4 – Water contact angle values of the films taken 180 s after the drop deposition.

Films	Water contact angle (º)
1	0.0 ± 0.0^{a}
2	$35.3 \pm 0.7^{b,c}$
3	0.0 ± 0.0^{a}
4	47.1 ± 1.3^{b}
5	28.0 ± 0.6^{c}
6	21.3 ± 0.6^{c}
7	$29.3 \pm 1.6^{b,c}$
8	21.9 ± 18.9^{c}
9	0.0 ± 0.0^{a}

$^{a-c}$ Different letters indicate statistical differences between samples (p<0.05).

Film water vapor permeability (WVP) is a key property to understand the moisture exchange between the coated product and the surrounding environment. Film WVP is a decisive factor in the understanding of moisture exchanges between the coated product and the surrounding environment. Table 5-5 shows the WVP values of the films.

Comparing samples films 3 and 4, the WVP value of sample 3 is lower than the WVP value for sample 4. This can be explained by the fact that the films 3 and 4 presented different alginate and glycerol concentrations (despite having the same amount of phenolic compounds). The low glycerol concentration in the film 3 and the decreased water affinity to the film due to the interaction between glycerol and alginate are the two main explanations for the lower WVP values (Boura-Theodoridou et al., 2020). Thus, as expected, an increase in glycerol concentration in the sample led to an increase in WVP value.

Comparing with the literature (Bierhalz et al., 2014; Lopes et al., 2017), the WVP of films are within the average value range for the matrices used (alginate and glycerol). The differences observed may be caused by different film preparation conditions, such as biopolymer and plasticizer type and measuring WVP conditions like temperature and relative humidity gradients. Many factors can affect the films' WVP, such as film thickness, water sensitivity and crystallinity. According to Jouki et al. (2013), high-crystalline polymers, such as alginate, are generally less

permeable due to their ordered structure and the mass transfer of a gas in a semi-crystalline polymer is mainly a function of the amorphous phase. Based on research by Mahcene et al. (2020), the decrease in permeability can happen due to the formation of hydrogen bonds between the alginate matrix and oxygen atoms of bioactive compounds. These interactions can limit the availability of hydrogen groups to form hydrophilic bonds with water and then lead to a decrease in the film's affinity for water.

Solubility of films in water may also provide insight on the behaviour of a film in an aqueous environment and it is a measure of its water resistance. This is also an important factor that determines biodegradability of films when used as packaging materials (Gnanasambadam et al., 1997). Table 5-5 shows the solubility and moisture content values obtained for alginate and glycerol films, incorporated with amaranth phenolic compounds.

Table 5-5 – Moisture content, solubility and WVP of the films.

Films	Moisture content	Solubility	WVP
	(%)	(%)	$(g \cdot (Pa \cdot s \cdot m)^{-1})$
1	72.06±0.52^{a}	93.88±1.21^{a}	2.56E-11±0.35^{a}
2	47.76±1.22^{b}	85.63±2.54^{b}	2.81E-11±0.24^{a}
3	72.13±0.68^{a}	98.86±1.98^{a}	3.70E-10±0.41^{b}
4	49.60±1.18^{b}	79.67±1.55^{c}	5.43E-10±0.22^{c}
5	74.68±0.55^{a}	97.74±0.82^{a}	5.15E-10±0.32^{c}
6	35.75±0.85^{c}	87.68±1.38^{b}	5.63E-10±0.28^{c}
7	53.57±0.82^{d}	97.78±0.71^{a}	3.08E-10±0.15^{a}
8	57.57±0.91^{e}	79.20±1.24^{c}	2.72E-10±0.32^{a}
9	62.02±1.87^{e}	67.05±1.95^{d}	2.66E-10±0.18^{a}

$^{a-d}$ Different letters indicate statistical differences between samples in the same column (p<0.05).

The increase in glycerol concentrations led to a significant decrease ($p < 0.05$) in the values of moisture content and solubility due to the hydrophobic nature of glycerol. The solubility also changed with the inclusion of phenolic compounds, significantly reducing their solubility at the

maximum concentration tested (1.5 %), explained by the increased hydrophilicity of the films with the addition of phenolic extracts.

5.3.4 Thermal Stability – Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC)

The glass transition temperature (T_g) is an important parameter in determining the mechanical properties of amorphous polymers (Biliaderis, Lazaridou, and Arvanitoyannis, 1999). Higher plasticizer concentrations led to the change of the physical structure of the films by decreasing the intermolecular forces between the polymer chains, increasing the free volume, and consequently increased the mobility of the polymer chains resulting in the lower values of T_g. T_g is the maximum temperature value at which amorphous phase chains acquire mobility. Below the T_g value, the polymer does not have enough internal energy to allow the displacement of one chain relative to the other, being in the glassy, rigid and brittle state. Above the T_g value, the chains rotate and move with the polymer in a more plastic state (Bonilla et al., 2013). In general T_g depends on the thermal history of the material, the molecular weight of the polymer chains, the presence of plasticizers, the degree of crystallinity and the composition of the sample (Peesan, Supaphol, and Rujiravanit, 2005), being specific for each material.

To allow the application of edible films in the food and pharmaceutical industries, comprehensive studies on thermal and stability characteristics of films are necessary as they may be subjected to thermal processes during their preparation, processing or consumption. The most used techniques to study the thermal stability of biopolymers are DSC and TGA (Neto et al., 2005). DSC is a conventional method of thermal analysis widely used to characterize the phase transition and it is used to measure the melting enthalpy (ΔH_m) and T_g of the films. These measurements are related to the crystallinity and texture of the films (Peesan et al., 2005; Sperling, 2001). The TGA and DSC results are shown in table 5-6.

Thermal analysis showed that films are stable up to 60 °C for all formulations (results not shown). The first stage (60–120 °C) can be attributed to water evaporation and chemisorbed water through hydrogen bonding (Mokrejs et al., 2009). The second stage (170-230 °C) is usually attributed to the presence of glycerol (Cerqueira, Souza, Teixeira, and Vicente, 2012). The third

stage at 230-330 °C (maximum peak in the DTG curve) is related to polysaccharide decomposition (Zohuriaan and Shokrolahi, 2004).

Table 5-6 - Enthalpy of melting (ΔH_m), glass transition temperature (T_g), onset temperature and TGA peak for alginate films with phenolic compounds.

Films	ΔH_m (J g^{-1})	T_g (°C)	***Onset temperature*** *(°C)*	***TGA peak*** *(°C)*
1	423.94±90.07^{a,b}	144.85±2.38^{a}	209.55±0.06^{a}	218.03±0.16^{a,d}
2	471.15±11.63^{a}	148.56±3.66^{a}	188.63±0.04^{b,c}	214.47±0.80^{b}
3	290.51±5.48^{c}	125.23±3.83^{b}	209.21±3.40^{a}	219.07±1.76^{a}
4	213.48±19.62^{c}	123.68±1.07^{b}	192.51±2.25^{b}	213.47±0.57^{b}
5	294.81±51.19^{c}	126.00±3.03^{b}	181.89±3.48^{c}	209.69±0.11^{c}
6	504.97±38.19^{a}	153.94±3.91^{a}	184.96±3.27^{b,c}	213.78±0.57^{b}
7	532.61±19.23^{a}	153.31±1.08^{a}	193.71±1.94^{b}	215.79±0.08^{b,d}
8	230.73±30.06^{c}	124.54±1.07^{b}	193.04±3.13^{b}	216.08±1.17^{a,b}
9	469.75±36.25^{a,b}	148.23±6.65^{a}	187.17±0.18^{b,c}	215.20±0.24^{b,d}

Note: Different letters in the same column indicate statistical differences between samples (p<0.05).

Regarding the presence or absence of plasticizing agents, glycerol has a great impact on T_g value due to its hydrophilic nature that allows a higher moisture content resulting in increased plasticizing ability (Benbettaïeb et al., 2019). It is expected that films with higher glycerol concentration will have a lower T_g value; however, it did not occur in this study. On the other hand, the ΔH_m and T_g value varied with different phenolic compounds concentrations. For instance, comparing samples 1 and 3 ΔH_m and T_g values, which have the same alginate and glycerol concentration but different phenolic compounds concentration, it can be observed that ΔH_m and T_g values of sample 3 were lower than sample 1 ($p<0.05$) because it presented higher phenolic compounds amount. The same behavior was observed for samples 2 and 4 ($p<0.05$). This could be due to phenolic compounds addition to film that reduced films crystallinity.

Thus, T_g of biodegradable films helps to choose the best storage conditions, and it is expected that WVP of the films will be higher at temperatures higher than the T_g value, where polymer chains are in greatest motion (Villarreal et al., 2013).

TGA curves also allows to obtain the onset temperature (i.e. start-up decomposition temperature), and the TGA peak (the temperature at which the film decomposition reaches maximum rate) (Table 5-6). Observing onset temperature and TGA peak values obtained for samples 5 and 8, these values were higher in sample 8 where glycerol amount is higher. This can be explained by the fact that glycerol depolymerization and pyrolytic decomposition, only occurs at a temperature of approximately 250 °C (Menzelet al., 2019).

5.3.5 Mechanical Properties – Young´s Modulus, Tensile strength and Elongation at break

Properties that reflect the ability of films to protect the integrity and durability of food products are mechanical properties including elongation at break (EB), Tensile Strength (TS) and Young´s Modulus (YM). Table 5-7 shows the values of these three properties that range according to the concentration of alginate, glycerol and phenolic compounds in the film. EB provides information on stretching the film, TS is the maximum stress that the film can withstand and YM corresponds to the elasticity of the film.

The mechanical properties of sample 6 have not been evaluated because it was not possible to handle this sample properly due to the large amount of glycerol and the small amount of phenolic compounds. On the other hand, sample 5 was the most rigid one, presenting high YM values, since it did not contain glycerol. These two types of films are not suitable for coating food products because one (sample 6) is too elastic and difficult to work with, and the other (sample 5) is a very rigid film that does not have the elasticity to coat a food product.

The EB value of films without phenolic compounds and with 0.5 % glycerol (sample 7) was 12.19±5.06 % and TS value was 1.12±0.25 MPa. The EB value films with 0.75 % of phenolic compounds and without glycerol (sample 5) was 2.20 ±0.69 % and TS value was 22.58±6.10 MPa. Comparing these samples, it is deduced that the ratio between glycerol concentration and phenolic compounds is involved in increased flexibility and the maximum stress that the film can withstand. Furthermore, comparing samples 1 and 2 which have the same phenolic compounds

concentration, being glycerol concentration higher in sample 2, the EB value increased when glycerol amount increased while YM and TS values decreased. This is because glycerol is a well-known plasticizer that increases polymer chains mobility and makes the films more extensible (Lahtinen et al., 2007).

Table 5-7 - Young´s Modulus (YM), Tensile Strength (TS) and Elongation at break (EB) for alginate films incorporated with phenolic compounds.

Films	**Mechanical Properties**		
	YM (MPa)	TS (MPa)	EB (%)
1	9.82±2.69^{a}	9.64±2.46^{a}	1.22±0.31^{a}
2	0.04±0.01^{b}	0.52±0.11^{b}	7.91±2.03^{b,d}
3	6.59±2.56^{c}	6.43±1.58^{a,d}	2.20±1.31^{a}
4	0.04±0.01^{b}	0.24±0.08^{b}	4.95±1.31^{a,b}
5	13.01±3.68^{a}	22.58±6.10^{c}	2.20±0.69^{a}
6	-	-	-
7	0.11±0.04^{b}	1.12±0.25^{b}	12.19±5.06^{c,e}
8	0.08±0.02^{b}	0.89±0.21^{b}	10.11±1.70^{c,d}
9	0.18±0.06^{b}	2.00±0.89^{b,d}	15.34±4.44^{e}

^{a,b,c,d} Different letters in the same column indicate statistical differences between samples (p<0.05).

5.3.6 Optical properties – Colour and opacity of the films

To evaluate the color and transparency of a film its optical properties are measured (Hutchings, 1999).

In general, films showed low opacity (OP) values (Table 5-8) and thus, good transparency, regardless glycerol, alginate and phenolic compounds concentration used in film formation. Packaging film transparency is a valuable property as it is an important feature in consumer choice.

The internal and surface film microstructure plays an important role in optical properties of the film (García et al., 2009).

Table 5-8 shows that OP value of the film 9 was higher than the values of the remaining samples. This can be justified by the fact that the concentration of phenolic compounds that forms these films correspond to the maximum concentration used. Thus, phenolic-rich films may be advantageous to decrease the incidence of light in a food product and consequently, decreasing light negative effects such as faster decomposition (Huber and Embuscado, 2009). Through visual observation, all films have a clear and smooth appearance despite slight differences in opacity values.

Table 5-8 - Color parameters (L, a* and b*), opacity and thickness of the alginate films incorporated with phenolic compounds*

Films	**Thickness**	**Opacity**	**Color Parameters**		
	(mm)	(%)	L*	a*	b*
1	5.03±1.01^{a}	9.86±0.87^{a}	95.26±0.31^{a}	0.00±0.02^{a,c}	4.08±0.21^{a}
2	5.00±0.26^{a}	7.80±0.53^{b}	94.26±0.28^{b}	-0.21±0.08^{b}	6.98±0.52^{b,d}
3	6.57±0.32^{a}	10.44±0.48^{a}	95.10±0.16^{a}	-0.06±0.09^{a,c}	4.76±0.30^{a}
4	6.43±0.65^{a}	7.60±1.93^{b}	94.13±0.34^{b}	-0.22±0-.12^{b}	7.56±0.49^{b}
5	6.10±1.00^{a}	10.11±0.73^{a}	95.22±0.36^{a}	0.03±0.02^{c}	4.25±0.23^{a}
6	5.00±0.75^{a}	3.35±0.29^{c}	96.49±0.07^{c}	-0.09±0.02^{a,b}	2.93±0.23^{c}
7	5.47±0.86^{a}	5.31±0.42^{d}	93.97±0.29^{b}	-0.20±0.06^{b}	6.62±0.41^{d,e}
8	4.83±0.63^{a}	9.14±0.58^{a,b}	94.51±0.47^{b}	-0.07±0.07^{a,c}	6.06±0.91^{e}
9	4.73±0.85^{a}	12.09±0.51^{e}	95.44±0.23^{b}	-0.20±0.05^{b}	7.77±0.27^{b}

^{a,b,c,d} Different letters in the same column indicate statistical differences between samples (p<0.05).

The OP of a material is an indication of how much light passes through it. Usually, higher thickness values will increase OP values because it is more difficult to light pass through the material. Table 5-8 shows that the lowest thickness value corresponds to the lowest OP value (sample 6) and the highest thickness value corresponds to the highest OP value (sample 9)

(Wilhelm et al., 2013). However, thickness values did not change significantly (p>0.05) with different phenolic compounds concentrations in the films. In addition, the OP value also depends on the films' components. Thus, it is possible to detect an increase in OP values with an increase in the phenolic compounds concentration in the samples (Table 5-8). The same behavior can be observed between sample 2 and 3 where the OP value is higher in sample 3 once it represents the sample with the highest phenolic compounds' concentration.

Regarding color parameters, Table 5-8 shows the L*, a* and b* values. The brightness parameter (L*) provides a black to white scale (0 to 100), a* is the coordinate for redness and greenness and b * is the coordinate for yellowish coloration (Şahin et al., 2009). The color parameters L*, a*, b* revealed the effect of alginate, glycerol, phenolic compounds on film color. The incorporation of glycerol in alginate films led to L* and a* values decrease, indicating a decrease of the lightness and an increase of the greenness of the film. This can be confirmed by comparing samples 4 and 5 (Table 5-8). The film sample 9 had a low a* value since, in addition to glycerol, it has also phenolic compounds. According to other authors (Corrales, Han, and Tauscher, 2009), the presence of phenolic acids and flavonoids due to addition of grape seed extracts in pea starch films, led to brownish-orange film color formation (L* and a* values decreased and b* values increased). Regarding the sample 9, the incorporation of the highest concentration of phenolic compounds in alginate films led to a decrease of the parameters L* and a*, indicating a decrease of the lightness and an increase of greenness of the film. The increase in b* indicates yellow intensification in the films.

5.3.7 Scanning electron microscopy (SEM)

Representative SEM micrographs of films with different concentrations of phenolic compounds are shown in Figure 5-1.

The upper surface of sample 7, without phenolic compounds is very smooth (Figure 5-1a), whereas the film images corresponding to phenolic compounds concentration of 0.5 % (sample 1) and 0.75 % (sample 5 showed a homogeneous film surface with very small plates and roughness (Figures 5-1b and 5-1c). On the other hand, the surface of the film became less smooth as the concentration of phenolic compounds increased. Some phenolic compounds aggregates were found in sample 9 (1.5 %) (Figures 5-1d). These results were similar to other reported results

(Martins et al, 2013, Coelho et al., 2017, Albuquerque et al., 2017).

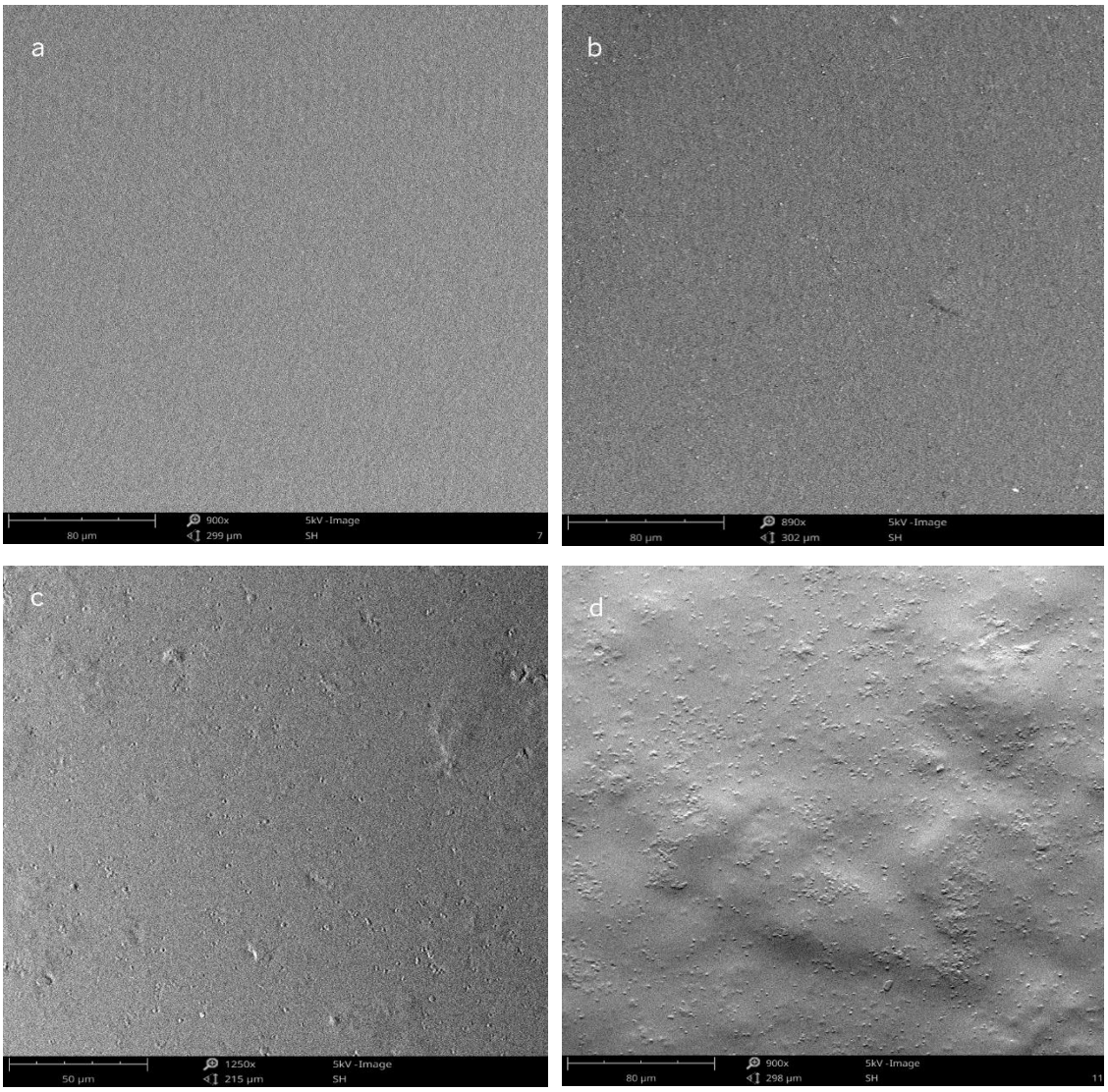

Figure 5-1 - SEM photos of the surface of alginate and glycerol films with (a) 0% (sample 7) (b) 0.5% (sample 1) (c) 0.75% (sample 5), (d) 1.5% (sample 9 of amaranth phenolic compounds.

SEM images showed that films had a rougher surface when the amount of phenolic compounds increased. These results can possibly explain the opacity results, because a gradually phenolic compounds concentration increase in the alginate film matrix alters the passage of light through the film, increasing opacity of the films.

5.3.8 Multivariate analysis using PCA, k-means cluster analysis and Pearson's correlation matrix

In this study, a multivariate technique (i.e., PCA) was used to analyze the relationship between the studied parameters of the films and their composition in order to better assess and explain statistically, the different behaviors observed in the studied films. Moreover, a k-means cluster analysis was performed to group the samples based on their similarities. The groups were identified in the PCA scores plot where samples with the same color represent the same cluster. The PCA biplot (i.e., scores and loadings plot) is represented in Figure 5-2.

It is possible to observe from the PCA that the principal component 1 (PC1) explains 51.40 % of the total data variability and the principal component 2 (PC2) explains 20.74 % of the total data variability. This implies that differences observed in the scores related to PC1 are more relevant when compared to differences observed in PC2. Furthermore, it is also possible to observe that the variations in the mechanical properties (YM, TS and EB), moisture content, alginate and glycerol contents are mostly explained by PC1 while variations in contact angle, TGA peak, WVP, phenolic compounds content, *Tg* and opacity are mainly explained by PC2.

From the analysis of the loadings plot it is possible to observe that the moisture content and the mechanical properties of the films are positively correlated with their alginate content and negatively correlated with their glycerol content. Therefore, samples with higher alginate concentrations can absorb more water and are less flexible (i.e., higher YM) but have higher tensile strength and withstand higher elongations (i.e., higher EB). However, it is also possible to observe that the films with higher glycerol concentration, despite improving the films flexibility by lowering its YM, can compromise its mechanical resistance (i.e., lower TS and EB).

It is widely reported that gel strength depends not only on the concentration of alginate and calcium and the degree of polymerization, but also on the source of the alginate, which confer the ratio of mannuronic and guluronic acid and different proportions. This ratio, and the way in which the acids are distributed in the alginate chains, have a marked effect on mechanical properties, and it is considered that alginates with a high proportion of guluronic acid produce rigid gels, which contribute to the gel formation with egg-box structure. The opposite holds for alginates with mainly mannuronic acids which produce softer, more elastic gels (Comaposada et al., 2015; Draget, Østgaard, and Smidsrød, 1990; S. Kim et al., 2018).

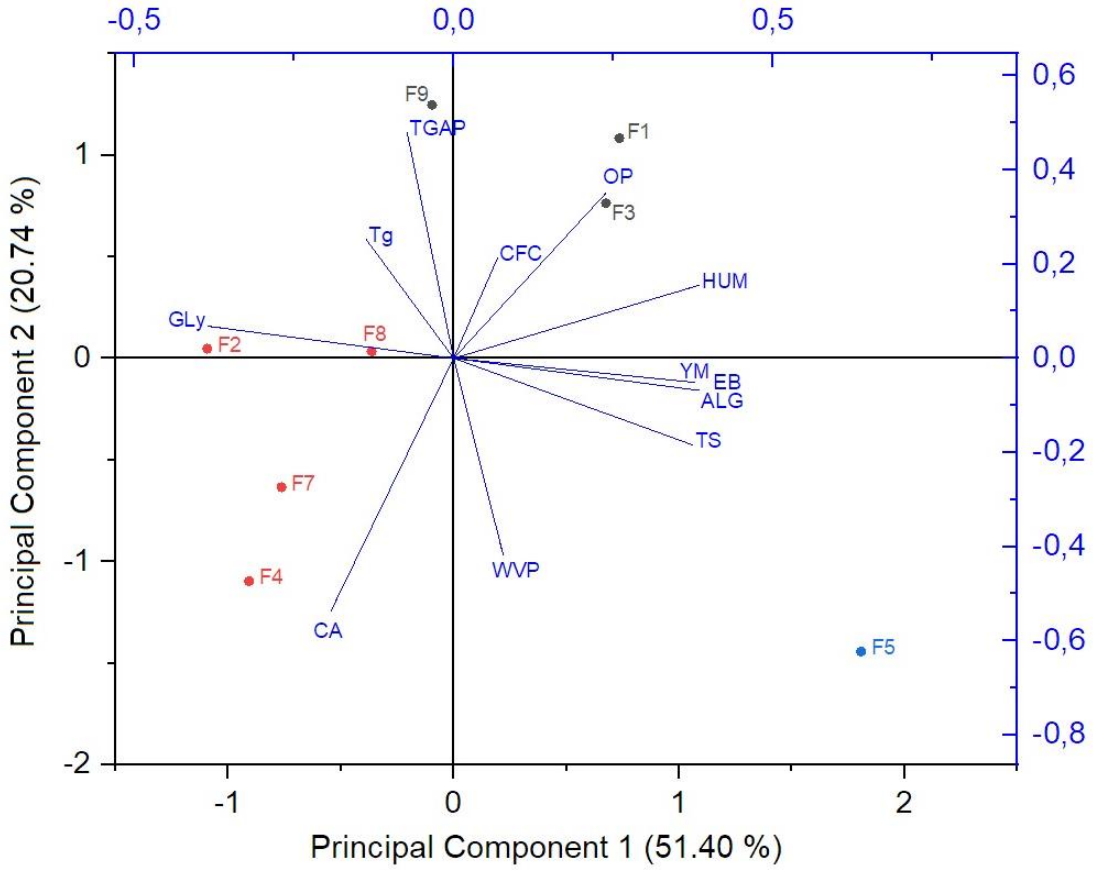

Figure 5-2 – Principal component analysis biplot between the first two principal components using k-means cluster analysis, where CA: contact angle; TGAP: TGA peak; ALG: alginate content; Gly: glycerol content; CFC: concentration of phenolic compounds; YM: Young's modulus; TS: tensile strength; EB: elongation at break; HUM: moisture content; T_g: glass transition temperature; OP: opacity. The dots represent the scores plot and the lines represent the loadings plot.

Regarding the thermal, optical, hydrophobicity and water permeability properties of the films, the PCA indicates that they are mainly correlated with the concentration of phenolic compounds indicating that higher concentrations of phenolic compounds, result in a more opaque and hydrophilic (i.e., lower water contact angle) film, with higher T_g and lower WVP. However, since PC2 only explains 20.74% of total data variability, these correlations must be carefully made.

A k-means cluster analysis was also conducted to group the different samples based on their similarity. This resulted in the formation of three distinct clusters (i.e., groups). A first group

containing samples F2, F4, F7 and F8; a second group containing F1, F3 and F9; a third group containing F5. In fact, samples within the same group present similar mechanical properties since no statistically significant differences ($p \geq 0.05$) were found in samples F2, F4, F7 and F8 regarding their YM and TS. Thus, less flexible but more mechanical resistant films can be produced by maintaining the same alginate/glycerol ratio (i.e., same amount of alginate and glycerol) with a concentration of phenolic compounds below 1%. On the other hand, more flexible but weaker films can be produced using higher alginate/glycerol ratio and higher phenolic compounds concentration.

To further assess how the different variables correlate with each other, specifically, how the different formulation components modulate the mechanical, thermal, hydrophobicity and optical properties of the films and which correlations are significant, a Pearson's correlation matrix was made and it is represented in Table 5-9.

Table 5-9 - Pearson's correlation matrix where the values marked as red correspond to significant correlation between the dependent and independent variables ($p < 0.05$).

	CA	TGAP	YM	TS	EB	HUM	T_g	OP
ALG	-0.4114	-0.2329	0.8707	0.9125	-0.4199	0.8964	-0.2518	0.4349
GLy	0.4114	0.2329	-0.8707	-0.9125	0.4199	-0.8964	0.2518	-0.4349
CFC	-0.3371	-0.0637	-0.0458	-0.0089	0.1151	0.2047	-0.3069	0.7982

CA: contact angle; TGAP: TGA peak; ALG: alginate; Gly: glycerol; CFC: phenolic compounds; YM: Young's modulus; TS: tensile strength; EB: elongation at break; HUM: moisture content; T_g: glass transition temperature; OP: opacity.

It is possible to observe that in fact, the YM and the TS significantly ($p<0.05$) correlate with alginate concentration (i.e., positive correlation) and with glycerol concentration (i.e., negative correlation). It is also possible to observe that the MC of the films also significantly ($p<0.05$) correlates with their alginate and glycerol content. The concentration of phenolic compounds significantly ($p<0.05$) correlates with films opacity which means that higher concentrations of phenolic compound increase this property. The color of the films changed with increasing phenolic compounds, the film became more opaque and brown in apparent color.

5.3.9 Fourier transform infrared (FTIR) spectroscopy

Infrared spectroscopy is a rapid and a non-destructive technique that has been widely used to characterize different polysaccharides (Jridi et al., 2019b). Also, FTIR spectroscopy is a powerful technique to investigate polymer blend miscibility. When chemical groups interact at the molecular level, changes are seen in FTIR spectra such as the shifting of absorption bands. These changes can be an indication of good miscibility of polymers (Martins et al., 2015). FTIR analyses were used to evaluate the interactions between alginate and glycerol with amaranth phenolic compounds (Figure 5-3).

The broadband ranging from 3500 to 3100 cm^{-1} was attributed to the OH elongation vibration formed by the hydroxyl group of polysaccharides and water and the wide band around 2800-3000 cm^{-1} was attributed to the CH elongation vibration (Cerqueira et al., 2011). The FTIR spectra of all samples also show a band in the 750-1300 cm^{-1} region that corresponds to the carbohydrate region (Figure 5-3).

The films showed characteristic bands at 3301.67 cm^{-1} (amide-A), 2939.59 cm^{-1} (amide-B), 1641.68 cm^{-1} (amide-I), 1547.66 cm^{-1} (amide-II) and 1242.95 cm^{-1} (amide-III). Amide A represents the stretching vibrations of N-H and/or O-H; amide B represents the C-H stretching and $-NH_2$ stretching; amide-I arises from C-O stretching vibration; amide-II arises from stretching vibration of C-N groups and bending vibration of N-H groups; amide-III relates to vibrations in plane of C-N and N-H groups of bound amide or vibration of CH_2 groups of glycine (Dou et al., 2018). As can be seen from Figure 5-3, amide-A and amide-B peaks were gradually shifted toward lower wavenumbers as the concentrations of amaranth phenolics increased. However, amide-I, amide-II and amide-III of films incorporating less than 0.75 % have not changed significantly.

Compared to film 7 (without phenolic compounds) and the alginate, amide-B wavenumber shifted toward lower wavenumbers as amaranth phenolics concentration increased. The changes in wavenumber are probably due to the interactions of -NH2 groups between peptide chains. The above changes indicated that the addition of amaranth phenolic compounds could cause changes in some functional groups.

Correspondingly, the FTIR results could better explain films physical properties changes.

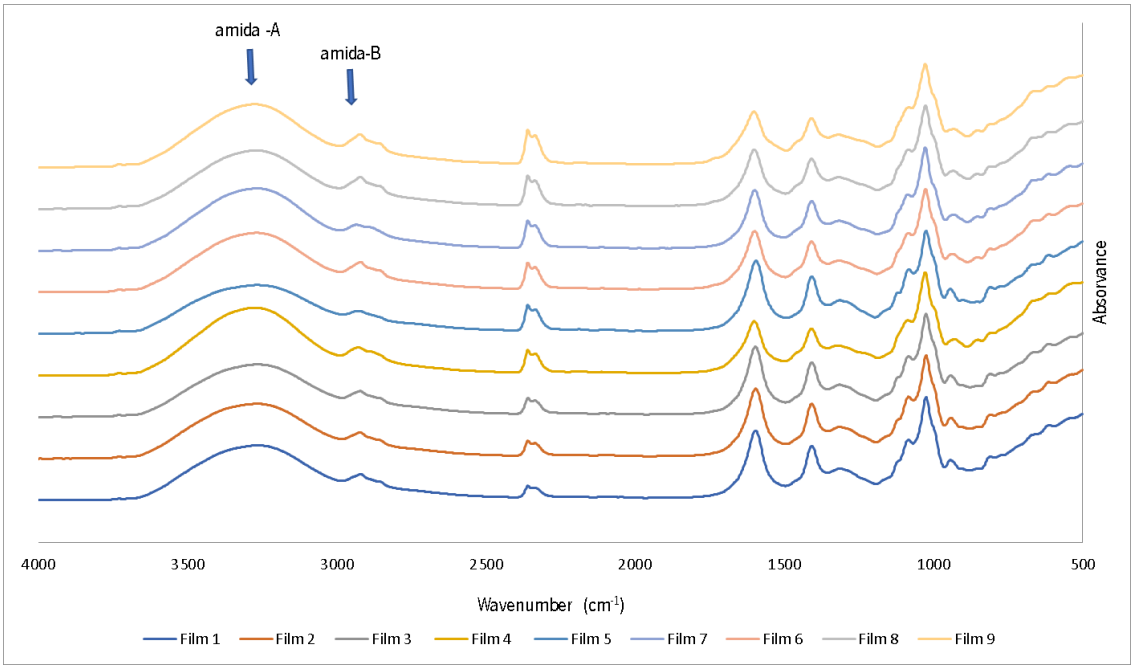

Figure 5-3 - Fourier transform infrared (FTIR) spectra of alginate films incorporating amaranth phenolic compounds

5.4 CONCLUSIONS

This study demonstrated that the amaranth phenolic compounds extraction using ethanol is adequate to obtain a product with high phenolic compounds retention and antioxidant capacity.

Amaranth phenolic compounds have been successfully incorporated in films composed of alginate and glycerol. The addition of phenolic compounds in these films resulted in intermolecular interactions, which was confirmed by FTIR spectra.

The results of this work provided useful information on the structural properties of alginate-based films with amaranth phenolic compounds and on the structural changes that occur in the film network induced by mixing different components proportions. Furthermore, this study can be used to tailor alginate films with amaranth phenolic compounds, depending on their final application. If higher mechanical stability is a priority, films with higher alginate/glycerol ratio should be produced. On the other hand, if the films flexibility is prioritized, the alginate/glycerol ratio should be equal to one.

Consequently, the incorporation of amaranth phenolic compounds in alginate and glycerol films is a good option for developing edible and environmentally friendly packaging to extend the shelf life of foods. Within practical application, the edible active films produced herein have broad perspectives.

In future studies it will be important to evaluate the antioxidant protection capability of the films in food simulants or food products through storage assessment and migration studies.

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SECTION IV

SYNTHESIS AND SUGGESTIONS FOR FUTURE WORK

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This chapter reports the main conclusions of the thesis and also presents suggestions for future work.

6.1 CONCLUDING REMARKS

The present thesis aimed at exploring amaranth grain compounds, in order to develop different systems (e.g. microcapsules and films) with functional properties to be applied in food products. Overall, important characteristics of components extracted from amaranth grain were evaluated and the result of this thesis provided clear indications about the possibility of developing new functional products based on amaranth polymers (starch and protein) and phenolic compounds. Therefore, this work contributes to the emerging effort of finding novel environmentally sustainable materials from renewable sources, such as amaranth grain which presents many unexplored nutritional and functional properties.

An overview of the main results achieved in this thesis can be summarized as follows:

- Amaranth starch- or protein-based microcapsules incorporating β-carotene were successfully produced by spray drying (CHAPTER 3). They showed good water solubility, moisture content and water activity when compared to other similar microcapsules found in the literature. Stability tests have shown that microcapsuled β-carotene is light sensitive but stable over time under dark storage. In addition, amaranth biopolymers could be a great choice for new capsules wall materials, because they guaranteed good β-carotene encapsulation efficiency.

- β- carotene release from protein and starch microcapsules in different media, β-carotene bioaccessibility, CAA and cytotoxicity using Caco-2 cells were studied (CHAPTER 4). It was demonstrated that microcapsules were stable under *in vitro* release tests on acidic food simulant medium but not on alcoholic food simulant medium during 30 h. It was also demonstrated that β-carotene incorporated in the amaranth starch and amaranth protein microcapsules were not cytotoxic to Caco-2 cells at 160 x dilution. β-carotene incorporated in amaranth protein microcapsules were not cytotoxic to Caco-2 cells. The microcapsules CAA has been shown to be 59.40 % for protein microcapsules and 69.27 % for starch microcapsules, indicating that the microcapsules exhibit the ability to protect cells against oxidative stress and β-carotene perform its antioxidant function. A better understanding of

how these microcapsules behave in the human GI tract was obtained, and β-carotene bioaccessibility increased when incorporated into amaranth starch and protein microcapsules. This study suggested that the developed amaranth-based microcapsules may be used as platforms for functional foods design with different functionalities, such as increased β-carotene bioavailability.

- The amaranth phenolic compounds extraction using ethanol is adequate to obtain a product with high phenolic compounds retention and antioxidant capacity. Alginate, glycerol and amaranth phenolic compounds have proven to be very promising films materials as good film formation capacity has been demonstrated (CHAPTER 5). In addition, this study has taken advantage of two widely used components in the food industry (alginate and glycerol) to develop new films with improved properties and thus, add value to this mixing system. Different alginate and glycerol concentrations have been shown to have an effect on film properties. The addition of phenolic compounds in alginate and glycerol films resulted in intermolecular interaction, which was confirmed by FTIR spectra. Furthermore, this study can be used to tailor alginate films with amaranth phenolic compounds, depending on their final application. However, phenolic compounds addition had an impact on films transparency, increasing their opacity. Therefore, different component proportions can be used to adapt edible films with enhanced mechanical and barrier properties. These findings suggest that, in addition to the potential antioxidant benefits provided, films containing phenolic compounds may also have good light barrier properties, which can potentially be applied to food products to preserve their quality (e.g., by preventing lipid oxidation) during shelf life.

In this context, this thesis has achieved its objectives of exploring amaranth grain components for potential novel food applications. Also, it may serve as a guide for the development of new funtional foods with desirable, profitable and nutritionally more elaborated physicochemical and functional properties using this pseudocereal.

6.2 FUTURE PERSPECTIVES

The work described in this thesis provided a proposal to use/explore different components of amaranth grain for food applications. However, several additional topics for further research can be raised by the present thesis, as follows:

- Test the amaranth biopolymer-based microcapsules with β-carotene in *in vivo* models to assess β-carotene bioaccessibility, bioavailability and toxicity;
- Incorporate amaranth biopolymer-based microcapsules with β-carotene into food products to fortify foods with β-carotene and verify its stability and reactivity during shelf life;
- Explore new phytochemicals to be incorporated into the amaranth biopolymer-based microcapsules developed in this thesis;
- Study the functionality of other amaranth components, such as squalene and tocopherols, to be used in biotechnology and nanotechnology applications, such as nanocapsules and films;
- Apply the films developed in this thesis to food products (for example, fruits and vegetables) and evaluate their functionality;
- Evaluate the films on an industrial scale to obtain a more accurate profile of their physico-chemical properties under industrial processing conditions;
- Product development and sensory quality studies should also be performed before any of the structures (i.e. microcapsules and films) described in this thesis are applied to commercial food products.