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Comparing the bioavailability properties of bee pollen and bee bread using an *in vitro* digestive system

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ABSTRACT

The consumption of natural products has increased significantly due to the idea that whether improving nutrition, improves health, general well-being and reduces the risk of developing certain diseases. Bee products, in special bee pollen and bee bread, have demonstrated several nutritional and bioactive properties, which make them functional foods par excellence. Both bee pollen and bee bread are natural products rich in lipid, protein, carbohydrates as well as minor components such as phenolic compounds.

Most of the bioactive properties are attributed to the powerful antioxidant and antiradical activity demonstrated by phenolic compounds. Nevertheless, bioactive claims are made without taking into consideration the further modifications to which phenolic compounds are subjected in the gastrointestinal tract. To determine and reveal the physicochemical parameter and the mechanisms of action of bioactive compounds in bee pollen and bee bread in the gastrointestinal tract, as well as the release of bioactive compounds from these bee products, determination of bioavailability properties and understanding their fate in the organism is crucial.

In this context, the purpose of the present work was to compare the bioavailability properties of bee pollen and bee bread using an *in vitro* digestive system, as well as the determination of the nutritional parameters. The research was based on a series of tasks that started with the collection of bee pollen and bee bread followed by the determination of physicochemical properties like total lipid, protein, soluble sugar, total carbohydrates content and energetic value. The second step was to determine how the bioactive compounds in bee pollen and bee bread are affected in each phase of the *in vitro* digestive system and their bioaccessibility score and stability. Finally, the changes in their antioxidant capacity were determined.

The study has shown that bee pollen and bee bread have different nutritional properties depending on their botanical origin. The results revealed that both bee products are rich sources of lipids, proteins and sugars, with high values in total carbohydrate and energy. The findings indicated a significant reduction in phenolic content in both bee pollen and bee bread samples at the end of gastrointestinal digestion compared to raw samples, and this was reflected in a decrease in their antioxidant capacity. Besides, the

bioaccessibility scores for total phenolic content were calculated on average 31% and 38% for bee pollen and bee bread, respectively, whereas the bioaccessibilityscore for total flavonoid content averaged 25% (bee pollen) and 35% (bee bread). The results showed that both bee products are highly affected by *in vitro* digestion.

In conclusion, this study clearly show that bee bread is either more accessible and richer in bioactive compound content compared to bee pollen, and both bee products have strong potential for widespread use in the food industry because of their rich macro and micronutrient content.

Keywords: physicochemical characterization; bioactive compounds; polyphenols; bioaccessibility; gastrointestinal tract; *in vitro* method

RESUMO

O consumo de produtos naturais aumentou significativamente devido à ideia de que, se houver melhoraria da nutrição, a saúde e o bem-estar geral aumentam, havendo uma redução do risco de desenvolver certas doenças. Os produtos apícolas, em especial o pólen de abelha e o pão de abelha, têm demonstrado várias propriedades nutricionais e bioativas, que os tornam alimentos funcionais por excelência. Tanto o pólen quanto o pão de abelha são produtos naturais ricos em lipídos, proteínas e hidratos de carbono, além de componentes em menores concentrações como os compostos fenólicos. Comparando com o pólen, o pão de abelha é caracterizado por um valor nutricional mais elevado, uma melhor digestibilidade e composição química mais rica.

A maior parte das propriedades bioativas é atribuída à elevada atividade antioxidante e anti-radicalar demonstrada pelos compostos fenólicos. Porém, estas características bioativas são aceites sem ter em consideração as modificações adicionais às quais os compostos fenólicos são sujeitos no trato gastro-intestinal. Torna-se fundamental, explorar e determinar os mecanismos de ação de compostos bioativos presentes no pólen e no pão de abelha e seu papel na prevenção de doenças, bem como a liberação desses compostos dos produtos apícolas, determinando a bioacessibilidade e biodisponibilidade durante a digestão no organismo.

Nesse contexto, este trabalho teve como objetivo comparar a biodisponibilidade do pólen e do pão de abelha através de um modelo *in vitro* de simulação da digestão gastro-intestinal, bem como a determinação dos seus parametros nutricionais. Para isso, o trabalho baseou-se numa série de tarefas que começaram com a obtenção das amostras de pólen e pão de abelha, seguida da determinação das propriedades físico-químicas como lipidos e proteínas totais, açúcares solúveis, teor de carboidratos totais e valor energético. Na segunda etapa determinou-se como os compostos bioativos no pólen de abelha e no pão de abelha são afetados em cada fase do sistema digestivo *in vitro* e o seu índice de bioacessibilidade e estabilidade. Finalmente, foram avaliadas as variações na sua capacidade antioxidante.

O estudo mostrou que o pólen e o pão de abelha têm propriedades nutricionais diferentes dependendo de sua origem botânica. Os resultados revelaram que ambos os

produtos apícolas são fontes ricas em lipídos, proteínas e açúcares, apresentando elevados de hidratos de carbono totais e energia. Apartir dos resultados verificou-se uma redução significativa no conteúdo fenólico em ambas as amostras de pólen e de pão de abelha no final da digestão gastro-intestinal em comparação com as amostras iniciais, e isso refletiu-se numa diminuição da sua capacidade antioxidante. Além disso, o nível de bioacessibilidade obtido para o conteúdo fenólico total foi em média de 31% e 38% para o pólen e pão de abelha, respectivamente, enquanto a bioacessibilidade para o conteúdo total de flavonóides foi em média 25% (pólen de abelha) e 35% (pão de abelha). Os resultados mostraram que ambos os produtos apícolas são altamente afetados pela digestão *in vitro*.

Em conclusão, este estudo mostra claramente que o pão de abelha é mais acessível e mais rico em conteúdo de compostos fenólicos bioativos, em comparação com o pólen de abelha, mas confirmando que estes produtos apícolas têm forte potencial para uso generalizado na indústria de alimentos devido ao seu rico conteúdo em macro e micronutrientes.

Palavras-chave: caracterização fisico-química; compostos bioactivos; polifenóis; bioacessibilidade; trato gastro-intestinal; métodos *in vitro*

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10-HDA	10-hydroxy-2-decenoic acid
CID	Collision-induced dissociation
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI	Electrospray ionization
DW	Dry weight
GAE	Gallic acid equivalent
GC	Gas chromatography
GIT	Gastrointestinal tract
HPLC	High-performance liquid chromatography
LC-DAD	Liquid chromatography coupled to diode array detection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
QE	Quercetin equivalent
RI	Refractive index
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated saliva fluid
TFC	Total flavonoid content
TPC	Total phenolic content
UHPLC	Ultrahigh-pressure liquid chromatography
UV	Ultraviolet

CHAPTER I

INTRODUCTION

1.1. Apiculture and bee products

Acquisition of bee products by humans, rock paintings from ancient times and historical finds show that apiculture has a very old history. Mesolithic rock painting from around 7000 BC at Spider Caves (Cuevas de la Araña) located in Valencia (Spain), shows a person collecting honey from bees. Wall painting on the Ancient Egyptian temple dating back to 2400 BC shows that beehives have been used for a long time and is seen that there is honey preparation (Mizrahi & Lensky, 2013).

Ancient writings from Egypt, Rome, Greece, and Orient, including Talmud, the Quran and the Bible, believed that honey and pollen were drugs for men, a substance that prolonged life and give pleasure, a source of youth and health (Fratellone, Tsimis, & Fratellone, 2016). Ancient people used honey and pollen to treat many diseases, including wounds, ulcers, and bowel problems while they used bee venom to treat gout and saw beeswax as a sacred object, but also useful in food, military weapon-making modeling and religious symbols (Mizrahi & Lensky, 2013; Morse & Root, 1990).

Apiculture began appropriately when people learned to develop some models in beekeeping and gain the ability to preserve the future of bee colonies with particular care and control, although important developments were not made in beekeeping practice and bee products until the 16th century (Crane, 2015). First, in 1717, it was found that the nectar collected by bees to make honey was produced by plants, and also ideas about the origins of various bee products were proposed, but the true origins of bee products were not known until a few centuries ago, and their detailed chemical composition was only determined in the late 1900s (Mizrahi & Lensky, 2013).

Honey bees belong to the genus *Apis*, which is a Latin word, meaning bee, and the api prefix is frequently used in beekeeping terms: apiarist (beekeeper) apiary (beehives terrain) apitherapy (use of bee products in the health), or apiculture (bee's breeding). Apiculture in a broader definition, is the maintenance of honey bees in hives and its management, mainly for obtaining honey and use for pollination, as well as the production of other bee products (pollen, bee bread, beeswax, propolis, royal jelly, and bee venom) and live material such as queen bees, swarm and packaged bees (Formato & Smulders, 2011). Beekeeping can be done in many places where plants and flowers are found, from the sea level, up to thousands of meters high, but it is always very important to adapt the beekeeping practices and the materials used for this purpose.

1.1.1. Honey

Honey is a natural substance with a complex composition produced by honey bees from the nectars collected from the flowers which they combine with their own special secretions. The composition, flavor, color, and taste of honey depend on geographical regions, climate, plant species visited by honey bees, processing and storage of honey (Escuredo, Dobre, Fernández-González, & Seijo, 2014). The main constituents of honey are carbohydrates such as glucose and fructose complemented by minor compounds such as proteins (mainly enzymes), amino acids, organic acids, carotenoids, aromatic substances, vitamins, and minerals (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Also, phenolic acids and flavonoids, which act as natural antioxidants with bioactive properties, can be found in honey (Tahir et al., 2017). Honey was used by early civilizations in traditional medicine, religious rituals, and other purposes. Nowadays, due to its biological and pharmacological properties, honey has many therapeutical applications for the treatment of health problems, such as skin infections in wounds and burns, treatment of ulcer, and as a protection against cancer and metastasis (Bogdanov, Jurendic, Sieber, & Gallmann, 2008).

1.1.2. Propolis

Propolis is a resinous substance collected by honey bees from various plants. The bee resin has a complex composition which varies with the region, plant species, soil, and climate. Propolis becomes sticky when heated but is very hard at low temperatures. Inside the beehive is used by bees to cover small cracks and to keep the temperature and humidity controlled, and also, thanks to its antimicrobial properties, to provide an aseptic environment and protection of the colony against diseases (Salatino, Teixeira, & Negri, 2005). The composition of propolis typically comprises plant resin (50%), beeswax (30%), essential and aromatic oils (10%) and the remainder 10% by pollen and other substances (Huang, Zhang, Wang, Li, & Hu, 2014). Propolis has a wide spectrum of biological-pharmacological activities (anti-cancer, antioxidant, anti-ulcer, anti-inflammatory, anti-fungal, anti-HIV, etc.), and it has been used in several medical applications such as the treatment of colds, wounds, heart diseases, diabetes, skin mycosis and stomach ulcer (Huang et al., 2014; Y. Li, Chen, Xuan, & Hu, 2012; Xuan et al., 2011).

1.1.3. Bee venom

Bee venom is an apitoxin produced by a gland in the abdominal cavity of the bee.

It is composed by a mixture of proteins (mainly phospholipase A2), peptides such as melittin, biogenic amines such as histamine, phospholipids, amino acids, non-peptide components, sugars and volatiles (Sobral et al., 2016). Since ancient times, humans have used bee venom for different purposes such as weapons manufacturing or the treatment of diseases (e.g., in the treatment of gout). Nowadays, bee venom is used in traditional medicine to treat different diseases, such as arthritis, rheumatism, pain, tumors, skin diseases, and multiple sclerosis (Hider, 1988; Mirshafiey, 2007).

1.1.4. Royal jelly

Royal jelly is a highly nutritional bee product, secreted by the hypopharyngeal glands of young worker bees and used to feed young bee larvae and the queen bee. Because bee larvae and the queen bee are fed directly with royal jelly, this product is not stored when secreted (Basa, Belay, Tilahun, & Teshale, 2016). The composition of royal jelly includes water, different proteins, sugars, lipids, such as the unsaturated acid 10-hydroxy-2-decenoic acid (10-HDA), a key molecule for the evaluation of royal jelly freshness, amino acids, organic acids, steroids, esters, phenols, minerals, and other components. The rich nutritional content of royal jelly varies according to external factors outside the colony (environmental conditions such as seasonal and regional conditions) but also due to biological factors related to bees (Sabatini, Marcazzan, Caboni, Bogdanov, & Almeida-Muradian, 2009). Royal jelly has been widely used commercially in many countries, particularly in cosmetics, due to its biological activities such as anti-inflammatory, anti-hypercholesterolemia, antibacterial, and antioxidant (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008).

1.2. Bee pollen and bee bread

Pollens are units of male gametophytes of flowering plants. Bees mix the pollen grains with their own secretions after collecting from the flowers; this process allows pollen to moisten and become pellets; then these pellets stick to the pollen basket on the hind leg of the bees and are transported to the hive (M. G. Campos et al., 2008). This new product, which bees collect from flowers and combine with their own secretions, is called bee pollen. Bee pollen is an important nutrient necessary for honey bees to grow on their larvae stage and to develop sufficiently during their youth (Calderone & Johnson, 2002). Beekeepers place pollen traps at the entrance of hives to obtain bee pollen collected by bees on flowers, and these traps enable the commercial collection of pollen (Figure 1).

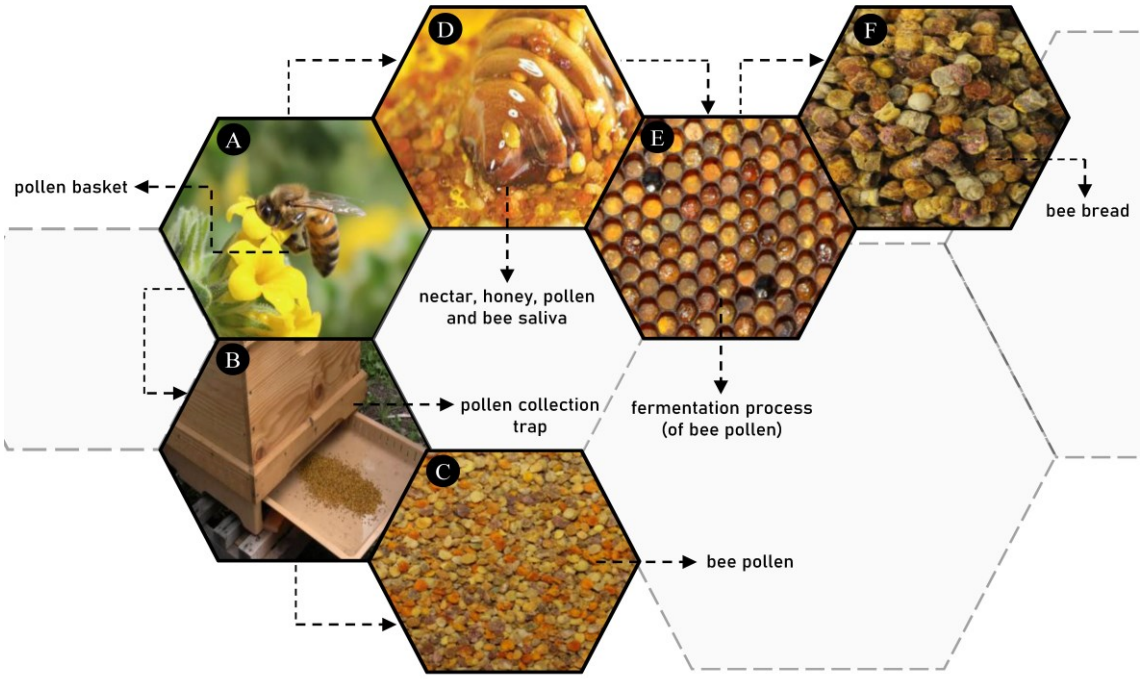

Figure 1. Process of bee pollen collection, including gathering pollen by bees (A), pollen traps placed at the entrance of beehives to collect pollen grains (B), obtained raw pollen grains (C) and the process of making bee bread, including gathering pollen by bees (A), combining bee pollen with honey, nectar and bee saliva (D), the process of lactic fermentation of pollen by bacilli of *Lactobacillus* bacteria in the combs (E), bee bread, which is a fermented bee product (F).

The next stage of collected pollen transported by bees is the storage in the cells of the combs of the hive. The stored pollen in the combs is mixed with the digestive enzymes secreted by bees, honey and organic acids and expose to lactic acid fermentation (Deveza et al., 2015), resulting the production of bee bread (Figure 1). In the process of transforming bee pollen into bee bread through a fermentation process, new products are also formed (Kieliszek et al., 2018), for example, pollen proteins are reduced to peptides and amino acids. Bee bread is a rich source of proteins, fats, and vitamins for the direct feeding of bees as well as the raw material for royal jelly production (Silici, 2019).

Bee pollen has been used by humans for centuries for food and health purposes. The ancient Egyptians described pollen as "life-giving dust", while the ancient Greeks believed it was "the food of kings that give youth and life". In ancient times, bee pollen not only played an important role as a product of rich nutritional value but also was used in the treatment of diseases and religious ceremonies (Mizrahi & Lensky, 2013).

1.2.1. Plant origin

Pollen are the grains contained in the male reproductive organs found in the anthers of high-flowering plants, and as it was previously mentioned, which bees collect and transform into bee pollen. Information about the botanical origin of bee pollen and bee bread is very important as an indicator of biological activity and commercial quality. The types of pollen in the bee pollen loads (the accumulation of pollen in the granular form) can be determined in two different ways: by microscopy or by chemical characterization through the phenolic profile analysis of the pollen. The first of these approaches can determine the plant family or genus rather than the species, whereas the second method can reveal the exact plant type (M. G. R. Campos, Frigerio, Lopes, & Bogdanov, 2010).

The pollen collected by bees may be different in color, size, appearance, biological activity, and physicochemical components. This difference is due to the genetics of the flower species and the geographical and climatic conditions of the areas that are visited by bees which have unique characteristics. Bee pollen is called monofloral when only one single botanical taxonomy characterize the pollen loads while preserving the physical and biochemical properties of the plant, whereas bees mix pollen loads from various flowers are called heterofloral (Carpes et al., 2013). In addition, if the pollen collected by bees is of anemophilous (wind-pollinated) plant origin, these pollen are lighter and dryer than those of entomophilous (pollinated by insects) plant origin, moreover, bee pollen originating from the entomophilous plants are characterized by higher nutritional value than anemophilous ones (Kieliszek et al., 2018).

1.2.2. Chemical composition

1.2.2.1. Water content

Fresh bee pollen loads contains 20-30% of water (M. G. Campos et al., 2008). Water content in bee pollen and bee bread is an important factor which affects the activity of microorganisms, deterioration, the shelf life of these products and many other parameters and so, for consumption, it is normally subject to dehydration The water content of dry pollen and bee bread can vary between 2% and 9% (Khalifa et al., 2020; Kieliszek et al., 2018). It is an important factor that conditions the amount of other components in this product and it causes unwanted odor and taste if the water content is less than 3%, due to discoloration and chemical reactions that can occur (Thakur & Nanda, 2020).

1.2.2.2. Carbohydrates

Carbohydrates are the main constituents and account for approximately 15–70% (w/w) of the dry weight (dw) of bee pollen and bee bread (Ares, Valverde, Bernal, Nozal, & Bernal, 2018; Kieliszek et al., 2018). The type of flower in which bees collect pollen as well as the geographic and climatic characteristics of the site of collection significantly affect the carbohydrate content of bee pollen and bee bread. Mono and disaccharides like glucose, fructose, sucrose, turanose, maltulose, and maltose account for about 94% of the total sugar in both bee pollen and bee bread (Khalifa et al., 2020).

Bee pollen also includes other carbohydrates such as polysaccharides, oligosaccharides, and dietary fiber. For example, cellulose, an important polysaccharide, has a content of about 3-4% in pollen and constitutes the primary component of the sheaths of pollen particles (Thakur & Nanda, 2020). On the other hand, oligosaccharides and polysaccharides are also important components that help regulate various biological functions in bee pollen and bee bread. Furthermore, these compounds are regarded as characteristic markers for discriminating the botanical origin of bee pollen (Q.-Q. Li et al., 2018). However, due to their high hydrophilic nature, high molecular weight and very similar polarities between these sugars, it is difficult to detect by both gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Martins, Morgano, Vicente, Baggio, & Rodriguez-Amaya, 2011).

1.2.2.3. Protein and amino acids

Bee pollen is generally characterized by high protein content, which is strongly dependent on the botanical origin of the pollen. The total protein content of bee pollen and bee bread is approximately 10-40% (w/w) of dw with 20 essential amino acids present (Q.-Q. Li et al., 2018). There is no much difference between the protein content of both bee products but bee bread has a higher level in terms of amino acids. This variability is due to the reduction of proteins to peptides and amino acids during the fermentation process of bee pollen, in consequence of the activity of the proteolytic enzymes that cause the cleavage of peptide bonds (Di Cagno, Filannino, Cantatore, & Gobbetti, 2019). Bee pollen and bee bread composition may also vary in certain amino acids such as tryptophan because some microorganisms can use them as energy sources for their metabolic activities (Kieliszek et al., 2018). In such cases, the concentration of these amino acids are reduced. Amino acids such as proline, glutamic acid, and aspartic acid are the major amino acids found in these bee products, especially in bee bread (Q.-Q. Li et al., 2018).

1.2.2.4. Lipids

Lipids are nutritious and important molecules that give energy and are necessary for the development of pollen. In bee pollen and bee bread, the lipid content is approximately 1-13% of dw and may include fatty acids, carotenoids, and sterols (Thakur & Nanda, 2020). The total content of unsaturated and saturated fatty acids in bee pollen are 62% and 38%, respectively (Seppänen, Laakso, Wójcicki, & Samochowiec, 1989). A total of 20 fatty acids were found in bee pollen, which mainly contained essential fatty acids such as linoleic acid, γ-linolenic acid, and arachidonic acid. Bee bread contains the same fatty acids as dry bee pollen, but both have a lower fatty acid content than fresh bee pollen collected by bees (Khalifa et al., 2020). As with other components (carbohydrate, protein, etc.), the concentration of lipids in bee pollen and bee bread depends on the botanical origin of the pollen.

1.2.2.5. Vitamins and minerals

Vitamins are a group of organic compounds with diverse biochemical roles and strong antioxidant activity. Bee pollen, rich in vitamins, containing almost all of them (0.02-0.7%), with vitamin B3, nicotinamide and niacin, as the most important among the B group vitamins (Q.-Q. Li et al., 2018). Furthermore, bee pollen contains carotenoids, such as β-carotene, which may be converted into vitamin A, with very good antioxidant activity. Bee pollen also contains vitamin E and small amounts of vitamin C. Bee bread is also rich in vitamins, among which C, B1, B2, B5, B6, B9, E, H, P, K and inositol (Khalifa et al., 2020). In addition, vitamin C and B6 concentrations are reduced in the conversion of bee pollen into bee bread.

Minerals are micro-nutrients necessary for proper functioning and regulation of metabolic pathways and physiological processes, and when taken inadequately, various health problems can occur. Both bee bread and bee pollen are a rich source of essential micronutrients containing several minerals: potassium, phosphorus, magnesium, calcium, sodium, sulfur, iron, copper, manganese, zinc, chromium, nickel and selenium (Ares et al., 2018). The mineral content of bee pollen and bread depends on the plant origin. Previous works described that minerals found in bee pollen may be used not only as dietary supplements but also as characteristic markers for defining plant origins and determining bee pollen quality (Q.-Q. Li et al., 2018).

1.2.2.6. Phenolic compounds

Phenolic compounds are secondary metabolites of plants which protect the plant against pathogens, ultraviolet, or stress factors. Since phenolic compounds have effects such as anti-hypertension, anti-hyperlipidemia, anti-inflammatory, anti-cancer and antioxidant, the interest in these compounds has recently been increasing. According to their chemical structure, phenolic compounds may be divided into flavonoids and phenolic acids. Phenolic acids include compounds such as gallic acid, chlorogenic acid, ferulic acid, caffeic acid, vanillic acid, syringic acid, benzoic acid, protocatechuic acid, cinnamic acid, and *p*-coumaric acid, while flavonoids include quercetin, isorhamnetin, apigenin, naringenin, kaempferol, catechin, epicatechin, luteolin, and hesperetin (Thakur & Nanda, 2020).

Bee pollen collected by bees from various plants is rich in polyphenolic compounds, ranging between 3-5% and highly dependent on the origin of the raw pollen (M. G. Campos et al., 2008). The accurate value may vary according to the method used to determine the phenolic and flavonoid content of pollen and the solvent in which the extracts were prepared (water, methanol, ethanol, etc.).

Flavonoids have an important place among polyphenols present in pollen. The chemical structure of flavonoids is characterized by the presence of a diphenylpropane ring system with a benzo-γ-pirone skeleton, and depending on its structure, seven flavonoids classes are distinguished: flavanones, flavanes, anthocyans, flavones, flavonols, isoflavones, and chalcones. Several flavonoids, in different forms, have been discovered during chemical research of bee pollen and bee bread, but the main found in bee pollen are kaempferol, quercetin, and isorhamnetin (Figure 2) (Rzepecka-Stojko et al., 2015). It has been shown by other studies that the bee bread contains flavonoids such as quercitrin, rutine, myricetin, and naringin, besides the flavonoids mentioned above (Čeksterytė, Kazlauskas, & Racys, 2006; Sobral et al., 2017; Tavdidishvili, Khutsidze, Pkhakadze, Vanidze, & Kalandia, 2014).

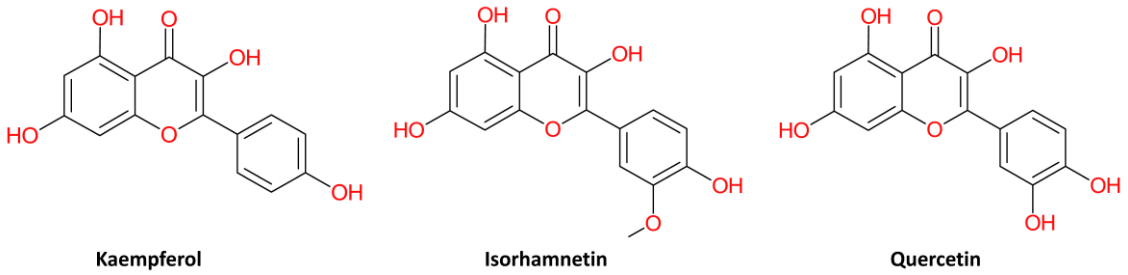

Figure 2. Structures of the main flavonoids of bee pollen and bee bread.

Another important group of phenolic compounds present in bee pollen are phenolic acids, which represents approximately 0.2% of bee pollen (Rzepecka-Stojko et al., 2015). They may have different structures and properties but contain an aromatic ring and a carboxyl group. Among the phenolic compounds, the derivatives of cinnamic and benzoic acids are of major importance due to the antioxidant activity of the phenolic ring, which is determined by the number of hydroxyl groups, the order of functional groups, and any steric effects caused by them (Rzepecka-Stojko et al., 2015). The most common phenolic acids in bee pollen and bee bread are chlorogenic, ferulic, cinnamic and caffeic acids (Almaraz-Abarca et al., 2004; Čeksterytė et al., 2006), as well as hydroxycinnamic and *o/p*-coumaric acids (Serra Bonvehi, Soliva Torrentó, & Centelles Lorente, 2001), benzoic acid derivatives (3,4-dihydroxybenzoic acid or protocatechuic acid; 3,4,5-trihydroxybenzoic acid or gallic acid) and 4-hydroxybenzoic acid ethyl ester (Čeksterytė et al., 2006; Chu, Tian, Jiang, & Ye, 2007).

In vivo and *in vitro* studies have shown that polyphenol compounds have beneficial effects on health by reducing the incidence of different diseases (cancer, diabetes, cardiovascular diseases, etc.) (Kroyer & Hegedus, 2001). The strong antioxidative properties of polyphenols are due to the presence of double bonds and the position of hydroxyl groups on the aromatic ring (Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006). Bee pollen and bee bread are characterized by their biological activity due to their rich polyphenol composition (Rzepecka-Stojko et al., 2015).

1.2.3. Potential effects of biological activities on health

The development and advancement of civilizations lead to new lifestyles and innovative food trends. The diet is shaped by various factors such as regional traditions, socioeconomic factors, cultural and educational activities. There is an intense relationship between diseases and the component content of consumed/used foods. Proper and adequate diet has a significant effect on physical and mental development, at the same time it has a protective effect against some of today's diseases. Since bee products such as bee pollen and bee bread contain almost all of the natural micro and macro-nutrients, the use of these products directly or indirectly in food plays important pharmacology and biomedical role in the prevention or reduction of some diseases (Basa et al., 2016).

In vivo and *in vitro* studies have shown that both bee pollen and bee bread have effective antioxidant activity, related to slowing or preventing oxidation from free

radicals, and so can be used as dietary antioxidants to prevent or reduce different diseases (Fatrcová-Šramková, Nôžková, Máriássyová, & Kačániová, 2016; Yıldız et al., 2013). The ability of bee pollen and bee bread to remove free radicals is closely related to the presence of phenolic compounds in their structure. The antioxidant effect of phenolic compounds is due to their properties such as scavenging free radicals, metal-chelating and preventing or reducing singlet oxygen formation (Rice-evans, Miller, Bolwell, Bramley, & Pridham, 1995). The loss of electrons in a substance is called oxidation, and oxidation reactions lead to the formation of free radicals. Excessive formation and accumulation of free radicals cause oxidative stress. The accumulation of oxidative stress in living organisms damages biomolecules such as proteins, DNA and lipids, and can cause many diseases such as hypertension, ischemia, neurodegenerative diseases, cancer, and rheumatoid arthritis (Halliwell, 2001).

The anti-inflammatory activity of bee pollen and bee bread is associated with components such as polyphenols and fatty acids. Previous studies on this matter found pollen to be effective against rat hind paw edema by inhibiting the production of cyclooxygenase-2 and nitric oxide (Maruyama, Sakamoto, Araki, & Hara, 2010). In another study, bee pollen extracts have been reported to have anti-inflammatory activity by reducing the production of nitric oxide and prostaglandins in mouse macrophages (Moita et al., 2014). In addition, bee bread has an inhibitory effect on acute and chronic inflammatory cascade compared to conventional nonsteroidal anti-inflammatory drugs (Khalifa et al., 2020).

Both bee pollen and bee bread are known to have anti-tumor activity. Some authors have focused their studied on the anti-carcinogenic activity of bee pollen and found to exhibit significant anti-proliferative effects on colon cancer cells (Wang et al., 2013). In another study, bee pollen has been reported to have an anti-carcinogenic effect against prostate cancer (Wu & Lou, 2007). In addition, some studies have reported that bee bread shows toxicity to the human tumor cell line (MCF-7, NCI-H460, HeLa ve HepG2) (Sobral et al., 2017).

One of the most investigated properties of bee pollen and bee bread is antimicrobial activity, and both have been shown to be effective against many microbes. The effect of Turkish bee pollen on 13 different pathogenic plant-bacteria was investigated and it has been reported that it has antibacterial effect against all studied pathogens (Basim, Basim, & Özcan, 2006). In addition, bee bread has been found to be effective against pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus,*

Bacillus cereus and *Pseudomonas aeruginosa* (Abouda, Zerdani, Kalalou, Faid, & Ahami, 2011). Bee pollen and bee bread have antimicrobial effect not only against bacteria but also against various fungi and yeasts (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis, Candida tropicalis, Geotrichum candidum* and *Rhodotorula mucilaginosa*) (Kacániová et al., 2012).

Another interesting feature of bee pollen is that it can reduce lipid levels in blood serum, that is, it plays an important role in the regulation of blood flow (Komosinska-Vassev, Olczyk, Kaźmierczak, Mencner, & Olczyk, 2015). Also, bee bread strengthens the immune system and gives resistance to many infections. Moreover, the bee pollen extract has stimulating effects on bone formation. The use of both bee pollen and bee bread in the treatment of burns is another biological activity of these products (Kieliszek et al., 2018). Additionally, bee bread regulates the functioning of the digestive system and plays an important role in the prevention of its disorders.

1.2.4. Side effects and allergic reactions

Bee pollen and bee bread are natural products of plant origin and are consumed by humans as food supplements or for other purposes. Normally bee pollen and bee bread are well tolerated by humans, however, there have been reports of some allergic reactions caused by bee pollen, including anaphylaxis (Greenberger & Flais, 2001; Jagdis & Sussman, 2012; Mansfield & Goldstein, 1981). These reports also include people suffering from hay fever. Allergic reactions to bee pollen of people without hay fever and taking bee pollen as food are low, and this ratio is similar to other foods. In a survey of bee pollen allergy in Polish beekeepers and their families, the following results were obtained: only 2 out of 493 beekeepers received negative reactions after pollen intake; only 22 cases of bee pollen intolerance were seen from the customers of beekeepers; 0.6% of bee pollen allergy occurred in family members of beekeepers (Basista, Filipek, & Sodzawiczny, 2012). More importantly, there have been no reported serious health problems or deaths due to the use and consumption of bee products, especially bee bread and bee pollen, to date.

1.3. Bioavailability of nutrients

Bioactive compounds are generally present in small amounts in foods, which have a positive effect on health by affecting physiological and cellular activities (Kris-Etherton

et al., 2002). It is very important to determine the mechanism of action of bioactive compounds in foods, their activity in preventing or reducing diseases, their release from the food matrix and the degree of absorption in the living organism (Stahl et al., 2002), and these issues are mainly defined under the heading of bioavailability.

Bioavailability can be defined as both uptake and metabolic use of a nutrient, which includes the following concepts: (i) bioaccessibility, (ii) absorption, (iii) tissue distribution and (iv) bioactivity (Figure 3) (Stahl et al., 2002). Bioavailability is closely related to the concepts of bioaccessibility and bioactivity and these two concepts will be discussed later. When the effect of bioactive compounds on human health is investigated, the bioavailability of these compounds is not always well known. Before these compounds become bioavailable, they must be released from food and modified in the gastrointestinal tract (GIT). Therefore, it is important to analyze whether the digestion process affects their stability, bioavailability and possible beneficial effects before determining any of their potential health effects (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014).

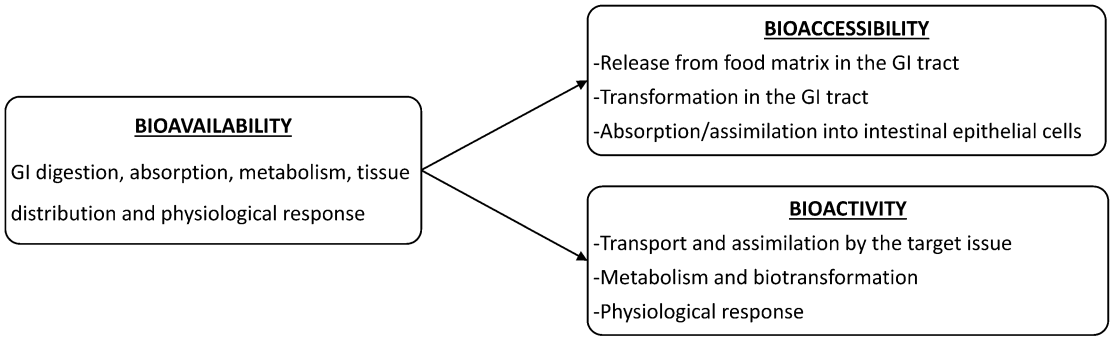

Figure 3. Brief description of bioavailability, bioaccessibility and bioactivity.

The bioavailability of bioactive compounds may vary due to their interaction with different nutrients such as less-processed food/beverages or polysaccharides in processed foods, as well as contact with the digestive system (Carbonell-Capella et al., 2014; Stahl et al., 2002). In a previously reported study, the use of food with medium-chain fatty acid triacylglycerols was found to affect (increase) bioavailability of the flavonol quercetin (Lesser, Cermak, & Wolffram, 2006).

1.3.1. Bioaccessibility and bioactivity

Bioaccessibility can be defined as the amount of nutrients available for absorption that is released from the food matrix in the GIT (Stahl et al., 2002). This term includes

digestion of foods in the GIT, nutrients ready for absorption, absorption/assimilation of intestinal epithelial cells and then presystemic, intestinal and hepatic metabolism (Carbonell-Capella et al., 2014). To assess the bioaccessibility of food, the GIT is usually simulated using *in vitro* methods and is sometimes followed by the use of Caco-2 cells (Vors et al., 2012).

Bioactivity, another concept closely related to bioavailability, is a broad concept and includes the transport of nutrients or bioactive compounds in living organisms, access to target tissues, interaction with biological molecules, biotransformation, and physiological response resulting from tissue uptake. Bioactivity measurements can be evaluated *in vivo*, *ex vivo* and *in vitro*. Although bioavailability and bioaccessibility are often used in a similar and unclear manner, it should be clarified that bioavailability includes either bioaccessibility and bioactivity (Etcheverry, Grusak, & Fleige, 2012).

1.4. The digestive process

The endogenous digestion process results in the progression of nutrients through the GIT, the breakdown of polymers into monomers by digestive secretions and absorption of monomers, water, and electrolytes into the bloodstream. This process works as follows: (i) small pieces of nutrients in the mouth are mixed with the saliva and pass into the stomach, (ii) the stomach makes the food semi-juicy with its own secretions and sends it to the small intestine and (iii) semi-juicy nutrients passing through the intestines are digested with the help of bile and pancreatic secretions and absorbed (Figure 4).

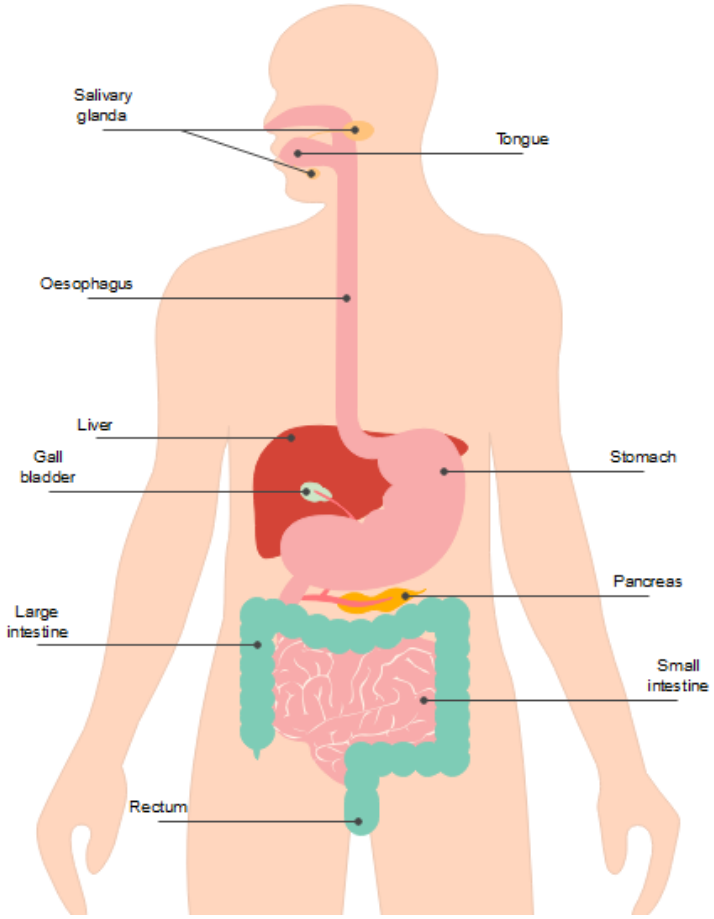

Figure 4. Gastrointestinal tract.

1.4.1. Oral phase of digestion

The digestive system starts in the mouth, and both chemical and mechanical digestion occurs in it. Saliva contains plenty of water, electrolytes, mucin, α-amylase and lingual lipase. Food is mixed with saliva containing α-amylase and lingual lipase enzymes when chewed in the mouth. The ptyalin enzyme, an α-amylase are secreted from the parotid gland and initiate the digestion of dietary carbohydrates, hydrolyzing them to maltose and glucose (J. Chen, 2009). Subsequently, when food reaches the stomach, α-amylase loses amylolytic activity with the acidity of gastric secretion (Pedersen, Bardow, Jensen, & Nauntofte, 2002), ie at pH $\leq$ 3. Furthermore, it is not known whether lingual lipase is important for the detection of oral fat in humans, but it is thought to contribute to the oro-sensory perception of lipids (Gambareli, Serra, Pereira, & Gavião, 2007).

1.4.2. Gastric phase of digestion

The human stomach is like a muscular bag, sealed both from the top and bottom, which facilitates the movement of food that is mechanically digested by the contractile

movements into the small intestine. After the oral phase of digestion is complete, the sphincter at the junction of the pharynx and the stomach opens and the food passes to the stomach (J. Chen, 2009).

Digestion of food in the stomach occurs in three steps: (i) firstly, gastric parietal cells secrete hydrochloric acid to facilitate hydrolysis of nutrients by the acid, and also allowing the conversion of pepsinogen to pepsin and activation of pepsin (Hinsberger & Sandhu, 2004), (ii) secondly, digestive enzymes such as pepsin and gastric lipase facilitate the hydrolysis of food (Sams, Paume, Giallo, & Carrière, 2016) and (iii) the food is mechanically digested with the contraction activity of the stomach (Lentle & Janssen, 2011). In the bottom part (antrum) of the stomach, the food is mixed and digested with the secreted enzymes and HCl, carried by antral movements and the digest is slowly discharged into the duodenum. In the passage of food from the exit of the stomach to the duodenum, it is neutralized with the help of bile and pancreatic secretions, while the controlled passage is provided. Gastric emptying of a solid diet is usually completed within 3 to 4 hours, but this time is shorter for an aqueous diet. The stomach nutrient content (carbohydrates, proteins, and lipids) and pH variation during the day, affect the gastric emptying time (Carrière, Renou, Ville, Grandval, & Laugier, 2001). After ingestion, the gastric pH rises, but with the release of HCl, the pH gradually decreases on a fast level (below pH 2) (Carriere et al., 1991), providing the pH required for enzyme activity.

1.4.3. Intestinal phase of digestion

The small intestine is one of the main organs of the digestive system and its main functional segments are duodenum, jejunum, and ileum. Although the small intestine has a diameter of 3-4 cm and a length of approximately 7 m, it has an enormous absorbent surface area. This absorbent surface is provided by the structure of the mucosa arranged in layers, and furthermore, this surface area is increased by plica circular, villi, and microvilli (Hinsberger & Sandhu, 2004). Small intestine motility is due to spontaneous and rhythmic contraction of the smooth muscles forming the intestinal wall. The primary purpose of small intestine movements is to mix and transport intraluminal contents (Hinsberger & Sandhu, 2004).

The primary functions of the small intestine are: production of enzymes and other components necessary for digestion, mixing and transport of intraluminal contents and absorption of nutrients. For the secretion of digestive secretions in the small intestine, the

intestinal mucous membrane needs to be mechanically and chemically stimulated, usually by the presence of chyme or food particles in the small intestine. One of the important sources of digestive secretion is the pancreas, which has exocrine-endocrine function. Pancreatic juice contains enzymes (pancreatic α-amylase, endopeptidases, carboxypeptidases, and lipolytic enzymes) that digest proteins, fats, and carbohydrates. While the exocrine pancreas secretes digestive enzymes into the small intestine, the endocrine pancreas secretes hormones that regulate glucose metabolism, primarily insulin and glucagon (Whitcomb & Lowe, 2007). Exocrine pancreatic secretion basically involves the function of facilitating digestion and protecting the digestive tract (Tsang, Cheng, & Leung, 2004). Bile salts, another important digestive secretion, are produced by the liver and stored in the gallbladder and secreted into the small intestine to aid digestion (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Bile acids are steroid acids that play two important roles in digestion. Firstly, bile salts have an emulsifying effect on fat particles, and secondly, they form small complexes with lipids called mycelium. Thus, bile salts contribute to the transport of fats and have an important role in the absorption of fat-soluble vitamins due to their effects on fat absorption (Ucok, Mollaoglu, Genc, Akkaya, & Sener, 2010).

1.5. *In vitro* digestion

There is a growing interest in the structural design of *in vitro* and *in vivo* models that simulate digestion processes to study the behavior of food and drugs in the human GIT. *In vivo* digestion methods that use animals or humans generally give the most accurate results but they can be time-consuming, costly and difficult to control, therefore much effort has been made to develop *in vitro* models to evaluate digestive effectiveness (Brodkorb et al., 2019). Since *in vitro* methods have the advantages of being physiologically relevant, faster, cheaper, reproducible, and lacking ethical constraints, it provides a useful alternative to human models by rapidly scanning food and drugs in the GIT. Nevertheless, if the properties of the digestive process such as temperature, residence time, secretion, mixing of digestive enzymes with food, transit time, pH, cellular properties and food disintegration are not properly combined, the results of many *in vitro* digestions may not be accurate (Hur, Lim, Decker, & McClements, 2011).

1.5.1. Methods used for *in vitro* digestion

Simulated digestion methods essentially take three steps into account: (i) digestion

in the mouth, (ii) digestion in the stomach, and (iii) digestion in the small intestine, and sometimes fermentation of the large intestine. These simulated methods attempt to mimic physiological GIT conditions taking into account their pH, digestion times, digestive enzymes, and salt concentrations.

In vitro digestion conditions may be changed according to the scope of the study. There are significant differences in the type of parameters tested in different models of digestion: chemical changes (hydrolysis of the main components in foods), location changes (release and adsorption of encapsulated or film-coated components), and structural changes (Hur et al., 2011). Most *in vitro* digestion methods make changes in the incubation times, the source of the enzymes used in digestion (enzymes obtained from human subjects, enzymes obtained from animal or plant sources, etc.) and pH of the mouth, stomach, and intestinal steps.

A blender is often used to simulate the disintegration of food in the mouth, but some *in vitro* methods require the help of human participants to simulate the first stage of digestion (chewing of food) (Woolnough, Bird, Monro, & Brennan, 2010). In another *in vitro* method, Caco-2 cell culture is used to simulate the absorption of bioactive compounds from food and drugs in the intestine (Vors et al., 2012). The properties and chemical components of foods are an important factor for the types and concentrations of enzymes to be used during *in vitro* digestion. Thus, the enzymes used should be adjusted according to the sample. Many *in vitro* digestion processes employ different enzyme species and concentrations based on different methods. In a study, it was found that the addition of enzymes to the *in vitro* digestion model did not significantly change the amount of catechin recovery from green tea (Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007). In another *in vitro* digestion model, similar results were obtained without using any enzyme in the digestion model established to determine the recovery of catechin (Record & Lane, 2001). Although many methods have been derived from previously reported *in vitro* methods, there are significant differences in the use of *in vitro* digestion parameters. In response to the need for standardization of *in vitro* models, a standardized static *in vitro* digestion method suitable for food has recently been published (Minekus et al., 2014). This accepted reported method is static and represents the enzymes and ionic conditions of lumen digestion. It does not yet contain the mechanical and dynamic components of a complete and valid *in vitro* digestive system.

In the literature, studies on how different components of bee pollen and bee bread are altered in the human GIT or on nutritive value are very limited, and there are a limited

number of studies applying a complete *in vitro* digestion model including oral digestion and all components of the digestive system. Zhou et al. (2018) studied the digestion of polysaccharides from bee pollen, and the *in vitro* digestion model they applied included human donors for oral digestion and fermentation phase, whereas the *in vitro* digestion model employed by Yesiltas et al. (2014) included only the gastric and intestinal phase. In a study on digestion of bee bread, enzymatic digestion (pepsin, trypsin, and papain) of bee bread was studied, although there was not a complete *in vitro* digestion (Nagai, Nagashima, Suzuki, & Inoue, 2005).

1.6. Aim and objectives

The main purposes of this work were: first, to determine the physicochemical parameters, bioactive compounds, and antioxidant properties of different bee pollen and bee bread samples; secondly, to apply a static and valid *in vitro* digestion model involving the oral, gastric, and intestinal phases; thirdly, to evaluate the antioxidant properties, bioavailability, and stability of phenolic compounds after digestion.

The aforementioned purposes were experimentally approached in the following manner:

- The determination of the plant origins of bee pollen and bee bread samples;
- The evaluation of the nutritional parameters of the samples, such as water, ash, lipid, protein, total carbohydrate, energetic value, and sugar profile;
- The determination of the total phenolic and flavonoid contents, the phenolic profile, and antioxidant properties.
- The application of an *in vitro* simulated digestion model and further evaluation of the stabilities of phenolics compounds and antioxidants.
- The comparison of the bioavailability properties of bee pollen and bee bread.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Ethanol methanol, sodium phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), potassium ferrocyanide ($C_6FeK_4N_6 \cdot 3H_2O$), trichloroacetic acid ($C_2HCl_3O_2$), acetonitrile (C_2H_3N), formic acid (CH_2O_2), sulfuric acid (H_2SO_4), diethyl ether ($C_4H_{10}O$), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), hydrochloric acid (HCl), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), petroleum ether, and gallic acid were purchased from Fisher Scientific (Pittsburgh, PA).

Folin-Ciocalteu's reagent, Kjeldahl catalyst tablets, potassium chloride (KCl), acetic acid glacial (CH_3COOH), ammonium carbonate ($(NH_4)_2CO_3$), and sodium chloride (NaCl) were purchased from Panreac Applichem (Barcelona, Spain). Iron (III) chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and naringenin were from Acros Organics (Pittsburgh, PA). Sodium carbonate anhydrous (Na_2CO_3) was purchased from Labkem (Barcelona, Spain).

Human salivary α-amylase (A1031-1KU), porcine pepsin (P6887), porcine pancreatin 4 × USP specifications (P1750), bile bovine (B3883), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D913-2), quercetin, *p*-coumaric acid, chrysin, fructose, glucose, saccharose, trehalose, turanose, maltulose, and maltose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol was purchased from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system (TGI pure system, Houston, TX, USA).

2.1.2. Samples collection and preparation

Three different bee pollen and bee bread samples were collected in August 2019 from *Apis mellifera iberiensis* hives located in different apiaries, in the northeast region of Portugal. The bee pollen samples, coded as BP-A1 BP-A2 and BP-A3, were collected in Bragança (Poulão), Bragança (Pinheiro Manso) and Vila Real, respectively, while the bee bread samples, coded as BB-A1 BB-A2 and BB-A3, were collected in Bragança (Poulão), Bragança (Pinheiro Manso) and Miranda do Douro (Póvoa), respectively (Figure 5). Bee pollen samples were removed from debris of wood and dead bee parts, while for bee bread, honeycombs were crushed manually and separated from wax. The samples were freeze-dried using a lyophilizer (FreeZone 4.5 model 7750031, Labconco, USA) and were stored at -20 °C for further analysis.

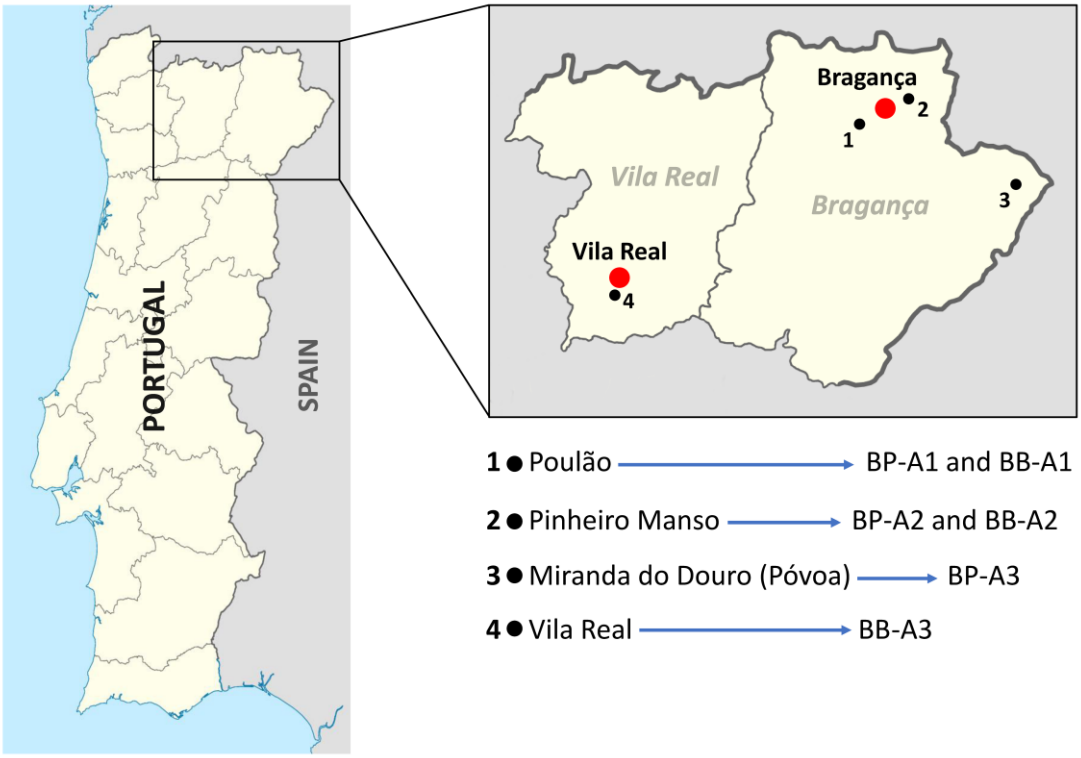

Figure 5. Geographical origin of the bee pollen and bee bread samples.

2.2. Methods

2.2.1. Palynological analysis

The homogenized bee pollen and bee bread samples (about 1 g) were placed in separate vials with distilled water and vortexed vigorously to soften the samples slightly. Then, 200 µL were taken for each sample from the resulting mixture and centrifuged at 1000 *g* for 5 minutes. The obtained pellet was subjected to acetolysis according to the method reported by Louveaux, Maurizio & Vorwohl (1978) and Von Der Ohe, Oddo, Piana, Morlot & Martin (2004). Pollen's identification and counting were performed using an optical microscope. More than 1200 grains per preparation were counted following the criteria of Vergeron (Vergeron, 1964).

2.2.2. Physicochemical analysis

Physicochemical analysis of the raw material, including water, ash, lipid, and protein was performed according to the procedures proposed by AOAC (1990). The water content was determined using a moisture analyzer (PMB 53 Moisture Analyzer, Adam Equipment, UK) following the AOAC 925.45 method. Ash content was determined by incineration (Optic Ivymen System) at 550 ± 5 °C according to the AOAC 923.05 method. The protein content (N × 6.25 for both bee products) of samples was estimated according

to the macro-Kjeldahl technique using the automatic Kjeldahl steam distillation unit (Pro-Nitro A, Selecta, Spain) following the AOAC 920.87 method. The lipid content was determined through the AOAC 989.05 method, using a Soxhlet apparatus and petroleum ether as solvent.

The estimated (by difference) total carbohydrate amount and total energy value of bee pollen and bee bread were calculated using the following equations, respectively:

$$Carbohydrates\ (g/100\ g\ dw) = 100 - (g\ ash\ +\ g\ lipids\ +\ g\ proteins) \quad (1)$$

$$Energy\ (kcal/100\ g\ dw) = 4\ \times (g\ proteins + g\ carbohydrates) + 9\ \times (g\ lipids) \quad (2)$$

2.2.3. Sugar profile analysis by HPLC

The sugar extraction procedure used was done accordingly to the previously described method (Tomás, Falcão, Russo-Almeida, & Vilas-Boas, 2017). Sugars were analysed by high-performance liquid chromatography coupled with a refractive index detector (HPLC-RI) in an integrated system with a pump (Knauer, Smartline system 1000) a degasser (Smartline 5000), an autosampler (Jasco AS-2057) and a RI detector (Knauer Smartline 2300). Data acquisition and remote control of the HPLC system was done by ClarityChrom software (Knauer, Berlin, Germany). The chromatographic separation was obtained with a column Eurospher 100-5 NH2 (4.6 × 250 mm, 5 mm, Knauer) kept at 30 °C. The mobile phase was composed of acetonitrile/water, 80:20 (v/v) at a flow rate of 1.5 mL min^{-1}.

The sugar profile was obtained comparing the retention times of standard individual sugars solutions. The quantitative assays were achieved using external calibration in a concentration range of 0.4 to 40 mg/mL, based on the following equations: fructose (y = 1,7219x + 0,4791; R^2 = 0,998), glucose (y = 1,5499x + 0,4068; R^2 = 0,999), saccharose (y = 1,8431x + 0,3705; R^2 = 0,999), trehalose (y = 1,7372x + 0,1899; R^2 = 0,999), turanose (y = 1,4418x - 0,1113; R^2 = 0,999), maltulose (y = 1,3132x + 0,012; R^2 = 0,998) and maltose (y = 1,1554x + 0,0555; R^2 = 0,993). The assay was performed in triplicate and the results were expressed as g/100 g dw.

2.2.4. Phenolic compounds extraction

The extraction process was performed according to the method outlined by Falcão et al. (2019). Briefly, 2 g of the pulverized sample was mixed with EtOH/H_2O (80:20, v/v, 40 mL) and stirred with a magnetic stirrer (Model: Multimatic 9-N, Selecta, Spain)

at room temperature for 6 h. The resulting mixture was filtered through a Whatman No. 4 filter paper, and the residue was re-extracted under the same conditions. The solvent was then vaporized with an evaporator (Rotary Evaporator model Hei-VAP, Heidolph, Germany) at 40 °C. Finally, bee pollen and bee bread extracts were lyophilized and stored at -20 °C.

2.2.5. Total phenolic content

Total phenolic content (TPC) was determined by the Folin-Ciocateu method using gallic acid as standard (Falcão, Freire, & Vilas-Boas, 2013). In the method, 0.5 mL of bee pollen ethanolic extract (1 mg/mL) was mixed with 0.25 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of 20% sodium carbonate was added and the final volume adjusted to 5 mL with deionized water. The resulting solutions were left in the water bath (70 °C) for 10 min and then cooled in the dark for 30 min. The same process was done for bee bread extract and soluble digestive fractions diluted to 1 mg/mL. Finally, the absorbance was read at 760 nm using a spectrophotometer (Analytikijena 200–2004 spectrophotometer, Analytik Jena, Germany). The TPC value was expressed as milligram of gallic acid equivalent per 100 g dry weight sample (mg GAE/100 g dw).

2.2.6. Total flavonoid content

The total flavonoid content (TFC) in the extracts was determined spectrophotometrically according to Falcão, Freire, et al. (2013). Briefly, 0.2 mL of the bee pollen ethanolic extract (5 mg/mL) was added to 0.2 mL of aluminum chloride solution (2% $AlCl_3$ in 5% glacial acetic acid in methanol). Then, 2.8 mL of 5% acetic acid/methanol solution was added to the mixture. After 30 min at room temperature, the absorbance was read at 415 nm. The same process was done for bee bread extract and digestive fractions diluted to 5 mg/mL. The TFC value was expressed as milligram of quercetin equivalent per 100 g dry weight sample (mg QE/100 g dw).

2.2.7. Antioxidant activity

2.2.7.1. DPPH radical scavenging activity

DPPH free radical scavenging activities of raw and digested samples were performed according to Brand-Williams, Cuvelier & Berset (1995) with slight modifications. 0.15 mL of sample with concentrations ranging from 0.03-0.43 mg/mL were added to 0.15 mL of DPPH and the absorbance was read at 515 nm using an ELX800

Microplate Reader (Bio-Tek Instruments, Inc.). The percentage of radical inhibition was calculated using the following equation:

$$\%\ Inhibition = [(A_{DPPH} - A_{Sample}) / A_{DPPH}]\ x\ 100 \quad (3)$$

The amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC_{50}) was achieved plotting the inhibition percentage against the extract concentration.

2.2.7.2. Reducing power activity

The Fe(III) reducing power activities of extracts was performed according to Oyaizu (1986). 0.25 mL ethanolic extract (1 mg/mL) of bee pollen was mixed with 1.25 mL phosphate buffer (0.2 mol/L, pH 6.6) and 1.25 mL 1% potassium ferricyanide, respectively. The mixture was incubated in a water bath at 50 °C for 20 min. Then 1.25 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 *g* (Centurion K2R series) for 10 min. 1.25 mL of the mixture was taken, followed by 1.25 mL of distilled water and 0.25 mL of 0.1% $FeCl_3$, and the absorbance was read at 700 nm. The same process was done for bee bread extract and digested samples. Gallic acid was used as standard and results were expressed as milligram of gallic acid equivalent per 100 g dry weight sample (mg GAE/100 g dw).

2.2.8. Phenolic profile by UPLC-ESI-MS

For the analysis, 20 mg of the previously extracted bee pollen and bee bread samples were weighed and dissolved in EtOH/H_2O (80:20, v/v, 2 mL). The raw sample extracts and the diluted soluble digestive fractions were filtered through a 0.45 μm membrane filter and kept at -20 °C until analysis.

A Dionex UltiMate 3000 ultra-pressure liquid chromatography instrument connected to a diode array and attached to a mass detector was used for LC/DAD/ESI-MS^n analyses (Thermo Fisher Scientific, San Jose, CA, USA). The analysis was conducted on a Macherey-Nagel Nucleosil C18 column (250 mm × 4 mm id; particles diameter of 5 mm, endcapped) and its temperature kept constant at 30°C. The conditions applied in the liquid chromatography were based on a previous work (El Ghouizi, El Menyiy, Falcão, Vilas-Boas, & Lyoussi, 2020); the flow rate was 1 mL/min, and the injection volume 10 μL. The final spectra data were accumulated in the wavelength interval of 190-600 nm.

The LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, CA, USA) equipped with an ESI source was set in the negative ion mode. The ESI conditions were the following: source voltage 5 kV; capillary voltage, –20 V; tube lens voltage, –65 V; capillary temperature, 325°C; and sheath and auxiliary gas flow (N2) 50 and 10 (arbitrary units), respectively.

Mass spectra was acquired by full range acquisition covering 100–1000 m/z. For the fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID). The normalized collision energy of CID cell was set at 35 (arbitrary units). The elucidation of the phenolic compounds was achieved by comparison of their chromatographic behavior, UV spectra and MS information, to those of reference compounds. When standards were not available, the structural information was confirmed with UV data combined with MS fragmentation patterns previously reported in the literature. All data acquisition was gathered using the Xcalibur® software (Thermo Fisher Scientific, San Jose, CA, USA).

Quantification was achieved using calibration curves for *p*-coumaric acid (0.00925-0.4 mg/mL; $y = 1.9 \times 10^7 x - 12927$; $R^2 = 0.996$), quercetin (0.037-1.6 mg/mL; $y = 4 \times 10^6 x - 10216$; $R^2 = 0.997$), kaempferol (0.037-1.6 mg/mL; $y = 4.3 \times 10^6 x - 13567$; $R^2 = 0.998$), chrysin (0.0185-0.8 mg/mL; $y = 1.2 \times 10^7 x - 51265$; $R^2 = 0.999$), and naringenin (0.0185-0.8 mg/mL; $y = 8 \times 10^6 x - 10998$; $R^2 = 0.998$). All compounds were quantified using the calibration curve of the structurally closest standard, and the final result was given in equivalent terms. Each value resulted from three different assays and is expressed as mg/g of sample.

2.2.9. Simulated *in vitro* gastrointestinal digestion

The *in vitro* digestion model was performed according to the method developed by the COST INFOGEST international network (Brodkorb et al., 2019; Minekus et al., 2014). This method consists of a sequential three phases: oral, gastric, and intestinal digestion. Before performing the *in vitro* digestion procedure, simulated saliva fluid (SSF), gastric fluid (SGF) and intestinal fluid (SIF) were prepared for each phase in which temperatures and pH were previously adjusted (Table 1). All enzyme solutions were freshly prepared and kept on ice.

Table 1. Preparation of stock solutions of simulated digestion fluids: SSF, SGF, and SIF stock solutions

			SSF		**SGF**		**SIF**	
			pH 7		pH 3		pH 7	
Constituent	Stock conc.		Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	g L^{-1}	**mol L^{-1}**	**mL**	**mmol L^{-1}**	**mL**	**mmol L^{-1}**	**mL**	**mmol L^{-1}**
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	—	—	11.8	47.2	9.6	38.4
$MgCl_2.6H_2O$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	—	—
For pH adjustment								
	mol L^{-1}		**mL**	**mmol L^{-1}**	**mL**	**mmol L^{-1}**	**mL**	**mmol L^{-1}**
NaOH	1		—	—	—	—	—	—
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2.2H_2O$ is not added to the simulated digestion fluids								
	g L^{-1}	**mmol L^{-1}**		**mmol L^{-1}**		**mmol L^{-1}**		**mmol L^{-1}**
$CaCl_2.2H_2O$	44.1	0.3		1.5*		0.15*		0.6*

* is the corresponding Ca^{2+} concentration in the final digestion mixture.

2.2.9.1. Oral phase

Briefly, 5 g of bee pollen and bee bread samples were weighed, transferred into 50 mL falcon tubes and mixed with 3.5 mL SSF stock solution. Then, 0.5 mL α-amylase solution of 1500 U/mL (made up in SSF stock solution), 25 µL of 0.3 mol/L $CaCl_2$ and 975 µL distilled water were added. The mixture was thoroughly mixed with a magnetic stirrer and adjusted to pH 7 with 1 mol/L NaOH and incubated in a water bath, in the dark, at 37 °C, for 2 min with constant shaking.

2.2.9.2. Gastric phase

After 2 min of oral phase incubation, 10 mL of oral bolus was mixed with 7.5 mL of SGF stock solution followed by 1.6 mL pepsin solution of 25 000 U/mL (made up in SGF stock solution), 5 µL of 0.3 mol/L $CaCl_2$, 0.2 mL of 1 mol/L HCl and 695 µL of distilled water. After adjusting the pH to 3, samples were incubated for 2 hours under the same conditions as in the oral phase. The pH of the samples was checked periodically during incubation to avoid pH changes.

2.2.9.3. Intestinal phase

In the final phase, gastric chyme was mixed with 11 mL of SIF stock solution, 5 mL of pancreatin solution of 800 U/mL made up in SIF stock solution based on trypsin activity, 2.5 mL of bile (160 mmol/L in fresh bile), 40 μL of 0.3 mol/L $CaCl_2$, 0.15 mL of 1 mol/L NaOH (to adjust the pH 7) and 1.31 mL of distilled water. NaOH or HCl (1 mol/L) was used to set the pH back to 7 and incubated under the same conditions as the gastric phase. During the incubation, pH was checked periodically to maintain pH 7. Finally, the obtained samples from each digestion phases were centrifuged for 15 min at 10000 *g* at 4 °C and the soluble and pellet fractions were stored at -32 °C until further analysis. Each bee pollen and bee bread samples were digested in triplicate and the replicates mixed.

2.2.10. Bioaccessibility index

Bioaccessibility (%) was described as the content of phenolic compounds released in the *in vitro* digestion process compared to the content of phenolic compounds in the tested samples, and the percentage of bioaccessibility was calculated according to the following equation (Leufroy, Noël, Beauchemin, & Guérin, 2012):

$$Bioaccessibility\ (\%) = \left(\frac{content\ of\ compounds\ released\ in\ the\ simulated\ digestion}{content\ of\ compounds\ in\ the\ tested\ sample}\right) x\ 100 \qquad (4)$$

2.2.11. Statistical analysis

All analyzes were performed in triplicate and all data were denoted as mean ± standard deviation (SD). The obtained data was analyzed using SPPS 26 software (Chicago, IL, USA) and GraphPad Prism version 8 (San Diego, CA, USA). One-way analysis of variance and the Tukey's multiple comparison test was conducted to see whether there is a statistical significance. $p < 0.05$ was considered as significant. Also, Pearson's correlation coefficients were calculated to ascertain the relationship between the tested parameters.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Botanical origin of bee pollen and bee bread

Forty pollen types were identified, at the species, genus, or family level, in the bee pollen and bee bread samples. To facilitate data analysis, only those with relative frequency percentages, higher than 0.1%, are presented in Table 2. A total of 17 pollen types were found at percentages greater than 3%, nevertheless, all samples were classified as heterofloral, since no major taxa were present at a relative frequency greater than 80% (M. G. Campos et al., 2008).

In the BP-A1 sample, *Plantago* sp. (47%) from Plantaginaceae family was the dominant pollen type, while *Crepis capillaris* (60%) from the Asteraceae family was the dominant pollen type in BP-A2 (Figure 6). For the BP-A3 sample, *Cytisus striatus* (48%) from the Fabaceae family was the dominant taxa. The presence of dominant taxon was not so evident for BB-A1 and BB-A3 samples. Instead, several different families had distribution at the accessory or isolated pollen level, with higher prevalence on *Cytisus striatus* (Fabaceae), *Castanea sativa* (Fagaceae), *Jasione montana* (Campanulaceae) and *Rubus* sp. (Rosaceae). *Castanea sativa* (48%) was the only dominant pollen type in the BB-A2 sample, followed by *Rubus* sp. with a relative frequency value of 22%. Asteraceae and Fabaceae families were described in previous studies as dominant pollens in bee pollen samples (n = 22) obtained from Douro International Natural Park in Portugal (Feás, Vázquez-Tato, Estevinho, Seijas, & Iglesias, 2012), which is in accordance to the results presently found. Besides, it is known that *Castanea* and *Rubus* pollen types are dominant in bee pollen and bee bread coming from the northern part of Portugal (Tomás et al., 2017). In another study, Morais, Moreira, Feás & Estevinho (2011) reported that the Fabaceae family was found in the bee pollen samples obtained from natural parks in Portugal. Even though the palynological results in the current study are in accordance with the works mentioned above, the pollen types in both bee products may vary depending on the collection season and in the flowers in the time of collection (Thakur & Nanda, 2020).

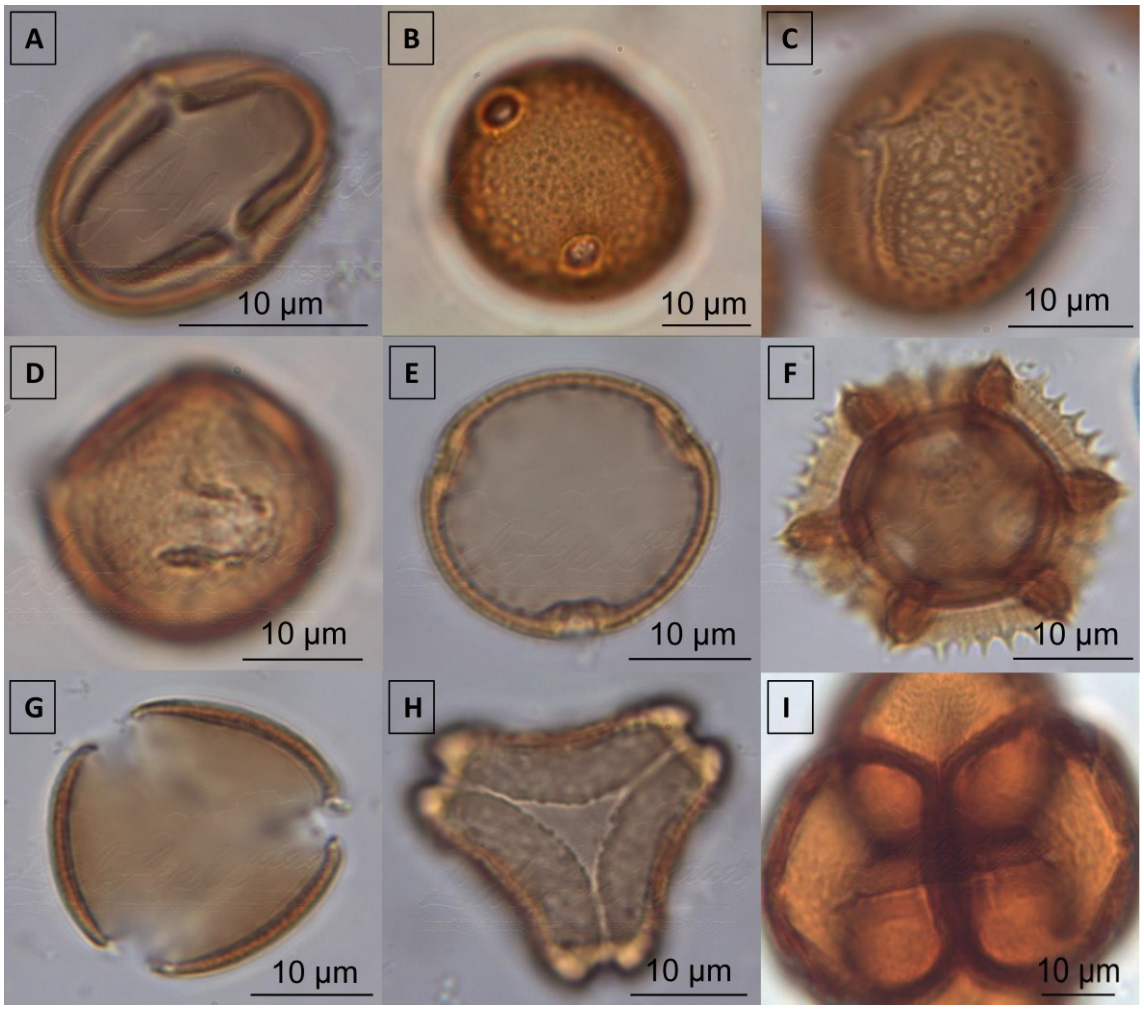

Figure 6. Microscopic view of some types of pollen seen in bee pollen and bee bread samples. *Castanea sativa* (A); *Plantago* sp. (B); *Trifolium* sp. (C); *Rubus* sp. (D); *Jasione montana* (E); *Crepis capillaris* (F); *Cytisus striatus* (G); *Eucalyptus* sp. (H); and *Erica* sp. (I).

Table 2. Relative frequency (%) of pollen types in bee pollen and bee bread samples.

		Relative frequency (%) of pollen types					
Family	**Pollen types**	**BP-A1**[a]	**BP-A2**[b]	**BP-A3**	**BB-A1**[a]	**BB-A2**[b]	**BB-A3**
Amaranthaceae	*Chenopodium* sp.	—	1.6	—	—	—	1.4
Apiaceae	*Eryngium*	1.0	—	—	—	—	0.1
	Thapsia vilosa	0.1	6.8	—	—	—	—
Asteraceae	*Centaurea* sp.	6.2	1.6	—	1.9	—	—
	Crepis capillaris	11.2	59.8	—	0.8	0.8	1.6
Boraginaceae	*Echium* sp.	8.8	0.6	0.7	4.2	1.5	—
	Pentaglotis sempervirens	—	—	—	1.0	—	—
Brassicaceae	*Raphanus raphanistrum*	—	—	3.8	0.4	0.1	1.9
Campanulaceae	*Jasione montana*	—	—	—	0.1	3.4	21.9
Crassulaceae	*Sedum* sp.	—	—	0.2	0.5	3.3	0.8
Ericaceae	*Erica* sp.	—	—	9.0	—	—	—
Fabaceae	*Lotus* sp.	0.1	—	—	1.1	—	—
	Cytisus striatus	—	0.1	47.7	6.2	6.5	19.7
	Trifolium sp.	5.9	0.2	1.7	12.2	6.5	—
Fagaceae	*Castanea sativa*	—	—	—	25.4	47.5	25.5
	Quercus sp.	—	0.3	—	—	—	7.0
Lamiaceae	*Lavandula* sp.	—	—	—	0.4	1.1	3.3
Myrtaceae	*Eucalyptus* sp.	—	—	24.1	—	—	—
Papaveraceae	—	0.3	0.2	3.5	0.3	2.2	0.6
Plantaginaceae	*Plantago* sp.	47.2	20.1	0.1	2.8	1.2	1.9
Poaceae	*Zea mays*	1.8	—	—	—	—	—
Resedaceae	*Sesamoides* sp. *or Reseda* sp.	—	—	1.9	0.1	0.2	—
	Rhamnus alaternus	—	—	0.1	1.0	—	—
Rosaceae	—	—	—	1.3	—	—	0.7
	Rubus sp.	0.6	—	2.7	37.3	22.3	10.2
Salicaceae	*Salix* sp.	—	—	1.3	0.5	0.8	—
Solanaceae	—	—	2.1	—	—	—	—
Classification		Heterofloral					

[a, b] The same letters represent samples collected in the same apiary. Dominant pollen (> 45%); Accessory pollen (15—45%); Isolated pollen (3—15%).

3.2. Physicochemical composition

As previously stated, the aim of this study was to evaluate the bioavailability of bee pollen and bee bread using an *in vitro* digestive system and especially focused on the changes that phenolic compounds undergo in the GIT and its impact on their bioavailability properties. In order to better understand the fates of the phenolic compounds in the GIT, it is important to determine the physicochemical parameters of both bee products, because macronutrients like proteins, lipids, and carbohydrates can interact with phenolic compounds in different ways and consequently may affect their bioaccessibility level (Carbonell-Capella et al., 2014).

The physicochemical parameters of bee pollen and bee bread samples are presented in Table 3 and Table 4. Even though they have similar nutritional parameters, there were statistically significant differences between bee pollen and bee bread, with exception for ash content and total carbohydrates. This may be due to the botanical origin of bee pollen, plant growth, or geographical conditions (Almeida-Muradian, Pamplona, Coimbra, & Barth, 2005; Carpes et al., 2013). On the other hand, the differences between bee bread samples were statistically (typically, $p < 0.05$) less compared to bee pollen. This means that bee bread samples have more stable values in terms of physicochemical parameters.

Table 3. Physicochemical composition (g/100 g of dw) and energetic value (kcal/100 g of dw) of bee pollen and bee bread samples.

Samples	Water content	Ash content	Lipid content	Protein content	Total carbohydrates	Energy
BP-A1	5.4 (±0.4)^{a}	3.8 (±0.5)^{a}	4.2 (±0.1)^{a}	21.1 (±0.7)^{a}	68 (±2)^{a}	406 (±1)^{a}
BP-A2	4.8 (±0.3)^{a}	2.7 (±0.1)^{b}	9.3 (±0.3)^{b}	15.1 (±0.4)^{b}	71 (±1)^{a}	436 (±2)^{b}
BP-A3	5.7 (±0.3)^{a}	3.4 (±0.2)^{a}	2.0 (±0.1)^{c}	29.1 (±0.8)^{c}	72 (±2)^{a}	396 (±0)^{c}
Average	5.3 (±0.5)	3.3 (±0.6)	5.2 (±3.7)	21.8 (±7.0)	70 (±2)	413 (±21)
BB-A1	7.7 (±0.8)^{b}	3.5 (±0.1)^{a}	2.9 (±0.3)^{d}	23.3 (±0.7)^{d}	72 (±2)^{a}	401 (±1)^{c}
BB-A2	7.8 (±0.4)^{b}	3.6 (±0.3)^{a}	3.1 (±0.3)^{d}	22.7 (±0.4)^{a,d}	71 (±2)^{a}	401 (±2)^{c}
BB-A3	7.4 (±0.9)^{b}	3.3 (±0.1)^{a,b}	4.8 (±0.4)^{a}	21.6 (±0.4)^{a}	71 (±1)^{a}	410 (±1)^{a}
Average	7.6 (±0.2)	3.5 (±0.1)	3.6 (±1.0)	22.5 (±0.8)	71 (±0)	404 (±5)

Results are expressed on the dry weight of bee pollen and bee bread, except water content. Each value is the mean ±SD, n = 3. In each column, different superscript letters (a–d) means significantly different ($p < 0.05$).

3.2.1. Water and ash content

The water content of bee pollen samples varied between 4.8 g/100 g (BP-A2) and 5.7 g/100 g (BP-A3), while bee bread samples were between 7.4 g/100 g (BB-A3) and 7.7 g/100 g (BB-A1). The water content of bee pollen samples was below the commercially proposed maximum limit (6 g/100 g) with an average value of 5.3 ± 0.5 g/100 g (M. G. Campos et al., 2008). There is no specified water content limit for bee bread, but compared to the reported studies, the water content of all bee bread samples was an acceptable level with an average value of 7.6 ± 0.2 g/100 g (Tomás et al., 2017; Zuluaga, Serratob, & Quicazana, 2015). The average ash contents for bee pollen and bee bread were determined as 3.3 ± 0.6 g/100 g and 3.5 ± 0.1 g/100 g, respectively. There was no statistically significant difference between both bee products and each sample, except for BP-A2. These values are within the range revealed by Kieliszek et al. (2018) and Zuluaga et al. (2015).

3.2.2. Lipid content

Lipids are an important macronutrient that forms the nutritional composition of bee pollen and bee bread, and it varies depending on the plant origin (Khalifa et al., 2020; Kieliszek et al., 2018). According to the obtained results, the highest lipid content in bee pollen samples was found in BP-A2 with a value of 9.3 g/100 g, while the lowest lipid content was determined in BP-A3 with a value of 2.0 g/100 g. It seems evident the richness of the *Crepis capillaris* pollen, compare to the others. The lipid contents of the bee bread samples were found to be 2.9 g/100 g, 3.0g/100 g, and 4.8 g/100 g for BB-A1, BB-A2, and BB-A3, respectively. There were significant differences between the minimum and maximum values of bee pollen samples, in contrast with the similar values found among bee bread samples. These values are within the proposed limits (1-13 g/100 g) of the bee pollen proposed quality standards (M. G. Campos et al., 2008) and are consistent with the results reported for bee pollen and bee bread in different countries (Almeida-Muradian et al., 2005; Kostić et al., 2015; Tomás et al., 2017; Zuluaga et al., 2015).

3.2.3. Protein content

Bee pollen and bee bread are characterized by high protein content which represents approximately 7-40% and 14-37% of their dry weight, respectively (Kieliszek et al., 2018). The maximum protein content among bee pollen samples was found in BP-

A3 (29 g/100 g), followed by BP-A1 (21 g/100 g) and BP-A2 (15 g/100). As mentioned before, variability between bee pollens is strongly dependent on the plant source from which the pollen is collected (Martins et al., 2011), and in this case, the pollen from *Crepis capillaris* was the poorest.

Regarding the protein composition of bee bread samples, the highest content was represented by BB-A1 with a value of 23 g/100 g but very similar to the other samples. There was quite a small difference between the average protein content of bee pollen (22 ± 7 g/100 g) and bee bread (23 ± 1 g/100 g) and not statistically significant ($p > 0.05$). However, in the multiple comparisons between both bee product samples, the differences were statistically significant, except for BP-A1 and BB-A2/BB-A3. These values are in accordance with the minimum (13 g/100 g, N x 6.25) composition requirements for bee pollen proposed by M. G. Campos et al. (2008). Additionally, in studies reported by Bakour, Fernandes, Barros, Sokovic & Ferreira (2019), Mayda, Özkök, Bayram, Gerçek & Sorkun (2020), and Nogueira, Iglesias, Feás & Estevinho (2012), the total protein is around 20% in both bee products.

3.2.4. Total carbohydrates and energy

The most abundant macronutrient in both bee product was carbohydrate. The total carbohydrate content averaged 71 g/100 g for both bee products, and there was no statistically significant ($p < 0.05$) difference between them. Representing the total carbohydrate content, these values include simple carbohydrates as well as complex carbohydrates such as pectin and cellulose, which forms the wall of pollen grains, and fiber. The energetic values of analyzed bee pollen and bee bread ranged from 396 ± 0 (BP-A3) to 436 ± 2 (BP-A2) kcal/100 g. These results are in the same range as those reported in previous studies (Nogueira et al., 2012; Tomás et al., 2017).

3.2.5. Sugar profile

The sugar composition of bee pollen and bee bread is given in Table 4. Accordingly, fructose and glucose were detected in all tested samples with values ranging from 18 to 24 g/100 g and 12 to 17 g/100 g, respectively, presenting the highest values compared to other sugars. The average fructose/glucose (1.86) ratio appears to be greater in bee bread samples compared to bee pollen (1.32). This may be explained by the addition of nectar during the bee bread processing, which is normally richer in the monosaccharide fructose, but also due to the use of glucose in the metabolic processes of

the lactic acid bacteria present in bee bread (Bakour et al., 2019), A similar situation was observed for sucrose in the samples, while bee pollen samples contain different ratios of sucrose, no sucrose was detected in the bee bread samples. The absence of sucrose in bee bread indicates that lactic acid bacteria prefer glucose as a primary source of nutrition. On the other hand, turanose, maltulose, and maltose were detected in all samples, except for BP-A3, but at lower levels than sucrose. The results in Table 4 show that there is a significant difference between the sugar content of the samples. Several studies reporting the sugar profile of bee pollen and bee bread showed a higher composition of fructose and glucose, followed by other sugars such as sucrose and maltose (Bakour et al., 2019; Belina-Aldemita, Opper, Schreiner, & D'Amico, 2019; Bertoncelj et al., 2018).

Table 4. Sugar profile of bee pollen and bee bread samples (g/100 g of dw).

Soluble sugars	BP-A1	BP-A2	BP-A3	BB-A1	BB-A2	BB-A3
Fructose	17.9 (±0.2)^{a}	22.0 (±0.7)^{b}	22.7 (±0.4)^{b,d}	23.5 (±0.4)^{c,d,e}	21.9 (±0.3)^{b}	22.6 (±0.6)^{b,e}
Glucose	13.6 (±0.1)^{a}	16.2 (±0.9)^{b}	17.5 (±0.1)^{b}	12.4 (±0.3)^{a}	12.2 (±0.2)^{c}	12.0 (±0.4)^{d}
Sucrose	5.7 (±0.0)^{a}	3.3 (±0.2)^{b}	11.2 (±0.1)^{c}	ND	ND	ND
Trehalose	ND	ND	ND	ND	ND	0.2 (±0.3)^{a}
Turanose	2.3 (±0.3)^{a,c}	3.2 (±0.1)^{b}	ND	2.3 (±0.2)^{a}	2.7 (±0.1)^{c}	2.5 (±0.0)^{a,c}
Maltulose	2.3 (±0.5)^{a}	4.2 (±0.2)^{b}	ND	2.3 (±0.2)^{a}	2.2 (±0.0)^{a}	3.5 (±0.3)^{b}
Maltose	1.1 (±0.4)^{a}	0.6 (±0.0)^{a}	ND	1.7 (±0.1)^{a,c}	1.2 (±0.1)^{a,c}	2.9 (±0.2)^{b}
Total	43.0 (±0.2)^{a}	49.5 (±0.7)^{b}	51.4 (±0.2)^{c}	42.2 (±0.2)^{d}	40.2 (±0.2)^{e}	43.6 (±0.3)^{a}

Results are expressed on the dry weight of bee pollen and bee bread. Each value is the mean ±SD, n = 3. In each row, different superscript letters (a–e) means significantly different ($p < 0.05$). ND = not detected.

3.3. Total phenolic and flavonoid content, and bioaccessibility level

The polarities, molecular weights, differences in chemical structures, and abundances of approximately eight thousand phytochemicals that exist in plants can be affected in different ways throughout the digestive process, and therefore their bioavailability may differ in the body (Liu, 2003). Along with this, the total quantity of phenolic compounds in food matrices does not reflect the amount absorbed by humans. The *in vitro* digestion method is a simple, fast, cost-effective with no ethical concerns, simulation process that may provide important data on the stability of the bioactive compounds throughout the digestion process. Also, the obtained *in vitro* digestion model results show a good correlation with the data obtained from *in vivo* studies (Bouayed, Hoffmann, & Bohn, 2011).

In the present study, the total phenolic content, TPC, and total flavonoid content, TFC, of undigested (raw) and digested bee pollen and bee bread samples are illustrated in Figure 7. The TPC for raw bee pollen samples ranged from 236 ± 9 (BP-A2) to 427 ± 12 mg GAE/100 g (BP-A3), while raw bee bread samples ranged from 317 ± 10 (BB-A3) to 378 ± 6 mg GAE/100 g (BB-A2). The TFC in bee pollen and bee bread ranged from 60± 6 to 272 ± 5 mg QE/100 g, in the following order: BP-A3 > BB-A3 > BP-A1 > BB-A2 > BB-A1 > BP-A2. The variation observed on the results were correlated to the different origin of the samples or the fermentation product in the case of the bee bread samples (Martins et al., 2011). Also, the TPC for the bee bread samples were more homogeneous compared to bee pollen, although the TFC had highly variable values. These findings are consistent with previous studies indicating that bee pollen and bee bread are an important source of phenolic compounds with antioxidant activity (Bakour et al., 2017; Borycka, Grabek-Lejko, & Kasprzyk, 2015; De-Melo et al., 2018; Zuluaga et al., 2015).

After the oral phase, a slight decrease was observed in the TPC of the digested BP-A1, BP-A2 and BP-A3 samples, with values of 192± 5, 114 ± 11 and 396 ± 14 mg GAE/100 g, respectively, Figure 7 (A). After the gastric phase, the TPC in samples decreased by 51% (BP-A1), 41% (BP-A2) and 69% (BP-A3). At the end of digestion, the TPC in BP-A1 decreased and reached 77 ± 8 mg GAE/100 g, while BP-A2 and BP-A3 increased slightly, reaching 70 ± 10 and 152 ± 6 mg GAE/100 g, respectively. The TPC of bee bread samples showed a similar trend to bee pollen after oral and gastric phases. However, a bigger increase was observed in TPC of all bee bread samples after the intestinal phase, and BB-A1, BB-A2 and BB-A3 reached 138 ± 2, 143 ± 10 and 112 ± 9

mg GAE/100 g, respectively. In addition, the TP content of each sample in each digestion phase was different from each other and was statistically significant ($p < 0.05$), except for BP-A2 and BB-A2 samples.

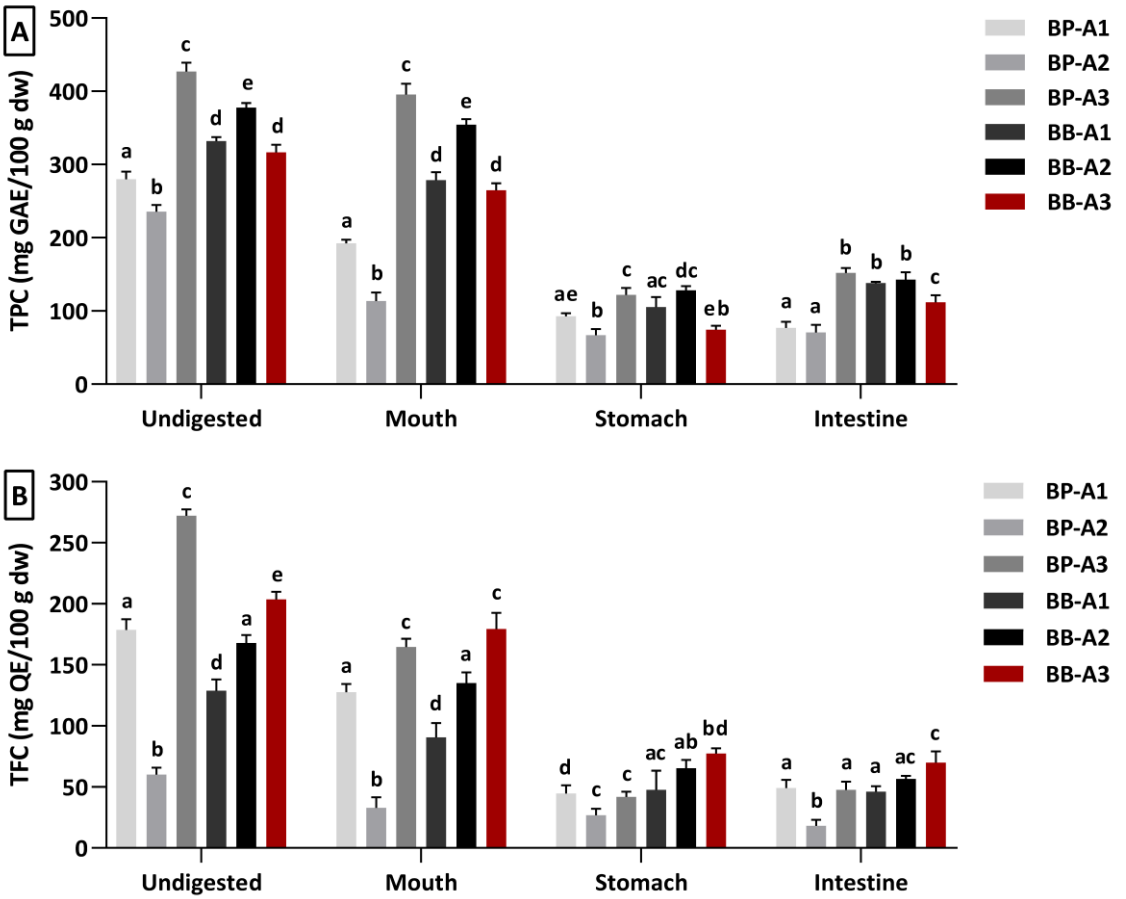

Figure 7. (A) The total phenolic content (TPC) and (B) total flavonoid content (TFC) of bee pollen and bee bread samples before (raw) and after each *in vitro* digestive phase.

Based on the values given in Figure 7 (B), the TFC in samples showed a decreasing trend after each digestive phase, except for BP-A1 and BP-A2 samples in the intestinal phase. Also, there was no significant difference in TFC between gastric and intestinal phases when compare for each sample. The TFC in bee pollen samples decreased by 77% on average, reaching 49 ± 7 (BP-A1), 18 ± 5 (BP-A2) and 48 ± 7 mg QE/100 g (BP-A3). A continuous decrease was also observed in the TFC in bee bread samples. After the intestinal phase, the TFC in BB-A1, BB-A2 and BB-A3 reached 46 ± 4 mg QE/100 g, 56 ± 2 mg QE/100 g and 70 ± 9 mg QE/100 g, respectively.

Based on the above results and calculating the bioaccessibility index, equation 4, the bioaccessibility level of bee pollen and bee bread samples at the end of the digestion

can be observed in Figure 8. The TPC bioaccessibility level of the BP-A1 and BP-A2 samples were 27 % and 30%, respectively, with no statistical difference between them, however with significant ($p < 0.05$) difference with BP-A3 (36%). The TPC bioaccessibility level for bee bread samples were calculated as 42% (BB-A1), 38% (BB-A2) and 35% (BB-A3), with an average value of 38% and higher than those of bee pollen, with statistical significance ($p < 0.05$). In respect to the TFC bioaccessibility, the highest score of bee pollen samples was found in BP-A2 with 30%, followed by BP-A1 (28%) and BP-A3 (18%), and for bee bread, the results were found to be 36% (BB-A1) and 34% for (BB-A2) and (BB-A3), again higher than bee pollen samples.

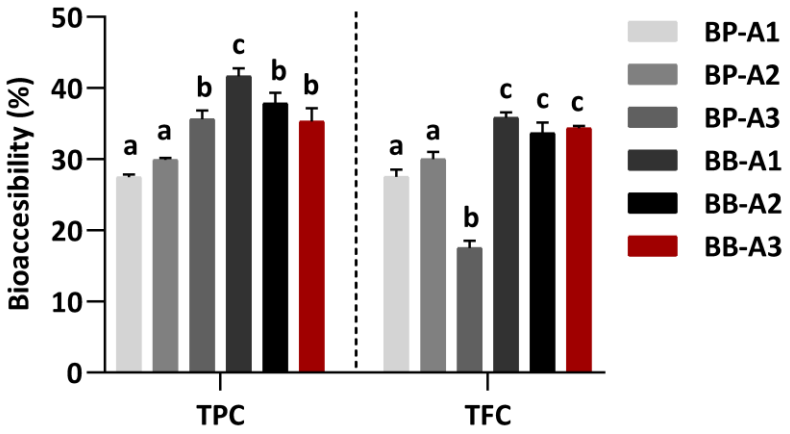

Figure 8. Bioaccessibility level (%) of total phenolic content (TPC) and total flavonoid content (TFC) in bee pollen and bee bread.

According to the obtained data, an overall decrease in the TPC of bee pollen and bee bread samples was observed at the end of the *in vitro* gastrointestinal digestion. Previous studies indicated that food matrices generally have a gradual decrease in their TPC as they pass through the digestive system (Ng & See, 2019; Schulz et al., 2017), however, there are also studies reporting a slight increase in TPC at the end of digestion despite a decrease in the oral and gastric phase for different foods (Kamiloglu, Pasli, Ozcelik, Van Camp, & Capanoglu, 2015; Quan et al., 2020), which was also observed in the current study, where the TPC of the samples was slightly increased in the intestinal phase compared to the gastric phase. This may be related to the multi-layered wall structure of the pollen grains which is resistant to digestive enzymes and pH changes. Also, the porous structure of the pollen grains may have contributed to the continuous release of phenolic compounds. Another important point is the high acidity of the stomach environment, which will have a strong effect on the released phenolic compounds (Ng &

See, 2019). Besides, the higher phenolic content released from bee bread samples comparing to the bee pollen can be explained by the partial digestion of the multi-layered structure of pollen grains by bacterial enzymes throughout the fermentation process of bee bread (Kaškonienė, Adaškevičiūtė, Kaškonas, Mickienė, & Maruška, 2020; Zhang et al., 2017). The findings in this study are in agreement with studies reported for TFC of different food matrices (Juániz et al., 2017; Ng & See, 2019; Yesiltas et al., 2014).

The TFC was generally found to be in a decreasing tendency, except for BP-A1 and BB-A3 samples in the intestinal phase. This decrease may be attributed to the breakdown of released and more accessible flavonoids by the action of digestive enzymes or different pH environment (Ng & See, 2019). In the study conducted by Pinto et al. (2017), it was reported that there was a decrease in the TFC of elderberries at the end of the digestion. Similar results have been obtained by other studies on edible mushrooms and carob flour as well (Ng & Rosman, 2019; Ortega, Macià, Romero, Reguant, & Motilva, 2011). These differences in TPC and TFC of the samples resulted in different bioavailability scores of the total quantity of compounds to be absorbed at the end of digestion. According to the findings, it can be said that bee bread generally has a higher phenolic content than bee pollen, as well as a more digestible product.

3.4. Phenolic profile and bioaccessibility level

The phenolic composition of both bee pollen and bee bread samples were characterized by LC/DAD/ESI-MS^n, in the negative ion mode.

The chromatographic profile, allowed the identification and quantification of 35 bioactive compounds, which included 21 phenolic compounds, mostly flavonols, and 14 phenylamides (conjugates of polyamines and hydroycinnamic acids), Table 5. The quantitative contribution of phenylamides were significantly higher than the other phenolic compounds.

Within the phenolic structures, flavonol derivatives such as quercetin, kaempferol, isorhamnetin and herbacetin glycosides, where the main compounds found in both bee pollen and bee bread samples. These flavonoids have also been observed in many reported studies for the phenolic content of both bee products, and were used for confirming our findings (El Ghouizi et al., 2020; Negri, Barreto, Sper, Carvalho, & Campos, 2018; Sobral et al., 2017; Su, Yang, Lu, & Liu, 2020). A few flavonoids such as kaempferol-*O*-rutinoside (*m/z* 593), quercetin-3-*O*-glucoside (*m/z* 463), quercetin-3-*O*-rhamnoside (*m/z*

447), hesperetin (*m/z* 301) and luteolin (*m/z* 285), were identified considering the retention time, UV-Vis profile, and MS pattern of the commercial standards.

The identification of the other compounds was done through interpretation of the fragmentation pathways detected in MS^n spectra comparing with that available in the literature and with combination with the spectral information from UV.

All tested samples typically contained flavonoid glycosides in their composition. The sugar moieties in the flavonoids were assigned to rutinosides, hexosides, glucosides and rhamnosides. These are the most common and frequent in nature and were now also confirmed in these samples. Methyl herbacetin-*O*-dihexoside *(m/z* 639), isorhamnetin-*O*-pentosyl-hexoside (*m/z* 609), kaempferol-3-*O*-rutinoside (*m/z* 593) and quercetin-3-*O*-glucoside (*m/z* 463) were identified as the most common compounds in both bee pollen and bee bread samples. BP-A3 sample was the richest among all in terms of diversity of flavonoids, at the same time it had the highest flavonoid content with a value of 2.70 mg/g. Quercetin-3-*O*-rhamnoside (*m/z* 447), isorhamnetin-*O*-deoxyhexoside (*m/z* 461) and luteolin (*m/z* 447) were only assigned in the BP-A3 sample and were the major compounds contributing to its high flavonoid content. The flavonoid content in bee bread samples was higher than the other two pollen samples, BP-A1 and BP-A2, with a great diversity of substances in different amounts, particularly, herbacetin derivatives such as methyl herbacetin-*O*-rutinoside (*m/z* 623), methyl herbacetin-3-*O*-hexoside (*m/z* 447) and methyl herbacetin (*m/z* 315). This assignment was supported by the previous identification of similar compounds in bee pollen and bee bread samples from *Brassicaceae* spp., *Asteraceae* spp., *Lavandula* spp. and *Plantago* spp. plant family (Anjos et al., 2019; Sobral et al., 2017; Urcan et al., 2018).

This study enable also the identification of an important group of compounds present in high concentrations in the bee pollen and bee bread samples, namely phenylamides and their derivatives. Phenylamides are derivatives of polyamines and are considered plant-specific secondary metabolites with a variety of functional roles with evidence on drought tolerance (Handrick, Vogt, & Frolov, 2010). Their presence has been established in seeds, flower buds, and pollen grains. Phenylamides are low-molecular products of covalent bonding between carboxylic groups of hydroxycinnamic acids and amine groups of aliphatic di- and polyamines or aromatic monoamines (Edreva, Velikova, & Tsonev, 2007). In both bee pollen and bee bread samples, the most widely distributed acidic parent compounds of phenylamides were caffeic, ferulic and *p*-coumaric acids while the aliphatic polyamines spermidine and spermine were found as the predominant

amine components of phenylamides. For the spermidines, the formation of the amide linkage between a phenylamide and phenolic acid can occur in the N^1, N^5 and N^{10} positions (Edreva et al., 2007). Spermine are mostly found conjugated with coumaroyl moieties in the positions N^1, N^5, $N^{10}$and N^4. These polyamine conjugates with phenolic compounds have a predominance in the plant species of several families such as Fabaceae, Asteraceae, Amaryllidaceae, and Araceae (Eliašová, Poracká, Pal'ove-Balang, Imrich, & Repčák, 2012).

Phenylamide compounds were detected in all tested bee pollen and bee bread samples at different concentrations and combinations. Its content varied broadly between 7.9 (BP-A1) - 38.7 (BP-A2) mg/g. N^1-acetyl-N^5, N^{10}-*di-p*-coumaroylspermidine (*m/z* 478), N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine (*m/z* 598), N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine (*m/z* 598), tetracoumaroyl spermine (*m/z* 785) and its isomers (*m/z* 785) were detected at different concentrations in all three bee pollen samples at very high levels. In bee bread samples, tetracoumaroyl spermine and its isomers were found in lower concentrations compared to bee pollen. However, bee bread samples exhibited a rich profile especially in respect to N^1, N^5, N^{10}-*tri*-caffeoylspermidine (*m/z* 630), N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (*m/z* 582) and N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer) (*m/z* 582), which can be related to the botanical origin of the samples. In a study conducted by Urcan et al. (2018), it was found that bee bread with origin in pollens from plant families such as Asteraceae and Fabaceae have a predominance of phenylamides. Also, it was reported that the pollens obtained from the Rosaceae family contain polyamine compounds (Strack, Eilert, Wray, Wolff, & Jaggy, 1990).

Table 5. Phenolic and phenylamide profile of raw bee pollen and bee bread samples.

t_R (min)	λ_{max} (nm)	$[M-H]^-$ m/z	MS^n (% base peak)	Proposed compound	mg/g raw sample					
					BP-A1	BP-A2	BP-A3	BB-A1	BB-A2	BB-A3
6.58	255, 349	771	MS^2: 609 (100); MS^3: 301 (100)	Quercetin-*O*-hexosyl-*O*-rutinoside[a,c]	0.08 ± 0.0	ND	ND	ND	ND	ND
7.48	257, 353	625	MS^2: 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin-diglucoside[a,e,h,]	ND	ND	ND	ND	ND	ND
8.43	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside[a,c,d]	0.04 ± 0.0	ND	0.31 ± 0.0	0.43 ± 0.0	0.33 ± 0.0	0.14 ± 0.0
9.94	265, 348	609	MS^2: 285 (100), 429 (49)	Kaempferol-*O*-dihexoside[a,d]	0.08 ± 0.0	ND	ND	ND	ND	ND
10.69	272, 326sh, 353sh	623	MS^2: 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside[a,c]	ND	ND	ND	0.09 ± 0.0	0.08 ± 0.0	0.02 ± 0.0
11.69	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside[a,e]	0.22 ± 0.0	0.07 ± 0.0	ND	0.06 ± 0.0	0.05 ± 0.0	0.06 ± 0.0
12.09	265, 348	593	MS^2: 284 (100), 285 (73), 429 (93)	Kaempferol-3-*O*-rutinoside[a,b,f]	0.02 ± 0.0	0.00 ± 0.0	0.03 ± 0.0	0.01 ± 0.0	0.02 ± 0.0	0.01 ± 0.0
12.62	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside[a,b,f]	0.01 ± 0.0	ND	ND	ND	ND	0.02 ± 0.0
13.31	256, 353	549	MS^2: 505 (100); MS^3: 301 (100), 300 (28), 463 (26)	Quercetin-*O*-malonyl hexoside[a,j]	ND	ND	ND	ND	0.01 ± 0.0	0.07 ± 0.0
13.61	270	477	MS^2: 315 (100), 462 (42), 300 (14); MS^3: 300 (100)	Methyl herbacetin-3-*O*-hexoside[a,c]	ND	ND	ND	0.04 ± 0.0	ND	ND
14.06	265, 347	447	MS^2: 285 (100), 284 (80)	Kaempferol-*O*-hexoside[a,k]	ND	ND	ND	ND	0.03 ± 0.0	0.16 ± 0.0
14.18	254, 347	447	MS^2: 301 (100)	Quercetin-3-*O*-rhamnoside[a,b,e]	ND	ND	0.81 ± 0.0	ND	ND	ND
14.31	254, 355	477	MS^2: 314 (100), 315 (45)	Isorhamnetin-*O*-hexoside[a,k]	ND	ND	ND	ND	0.01 ± 0.0	0.01 ± 0.0
14.44	255, 354	563	MS^2: 519 (100); MS^3: 315 (100)	Isorhamnetin-3-*O*-malonyl glucoside[a,h]	ND	ND	0.06 ± 0.0	ND	ND	ND
14.73	277, 311	301	MS^2: 283 (100), 286 (40)	Hesperetin[a,b]	ND	ND	0.05 ± 0.0	ND	ND	ND
14.99	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside[a,h]	ND	ND	ND	ND	0.07 ± 0.0	0.09 ± 0.0
15.39	299, 308	436	MS^2: 316 (100)	Di-*p*-coumaroylspermidine[a,i]	0.72 ± 0.0	0.20 ± 0.0	ND	ND	ND	ND

15.76	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	*N^1, N^5, N^{10}-tri*-caffeoylspermidine[a,d,g]	ND	ND	ND	0.20 ± 0.0	0.50 ± 0.0	ND
16.18	264, 341	431	MS^2: 285 (100)	Kaempferol-3-*O*-rhamnoside[a,c]	ND	ND	0.04 ± 0.0	ND	ND	ND
16.64	255, 354	461	MS^2: 314 (100), 315 (77), 299 (39)	Isorhamnetin-*O*-deoxyhexoside[a,c]	ND	ND	0.31 ± 0.0	ND	ND	ND
16.75	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	*N^1, N^5, N^{10}-tri*-caffeoylspermidine[a,d,g]	0.43 ± 0.0	0.08 ± 0.0	ND	1.53 ± 0.0	4.15 ± 0.0	0.51 ± 0.0
17.57	293, 314	644	MS^2: 358 (11), 482 (11), 508 (100); MS^3: 332 (27), 358 (100), 372 (49)	*N^1*-feruloyl-*N^5, N^{10}*-dicaffeoylspermidine[a,d,e]	ND	ND	ND	ND	0.04 ± 0.0	ND
18.09	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine[a,e]	0.21 ± 0.0	ND	0.38 ± 0.0	0.16 ± 0.0	0.13 ± 0.0	0.07 ± 0.0
18.18	299, 308	478	MS^2: 358 (100), 332 (12), 145 (5)	*N^1*-acetyl-*N^5, N^{10}-di-p*-coumaroylspermidine[a,f]	2.83 ± 0.1	0.69 ± 0.0	ND	ND	ND	ND
18.74	295, 311	614	MS^2: 478 (100), 468 (20), 452 (68), 342(5)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine (isomer)[a,e]	ND	ND	ND	0.06 ± 0.0	0.20 ± 0.0	ND
19.34	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine (isomer)[a,e]	ND	ND	1.36 ± 0.0	0.76 ± 0.0	1.98 ± 0.0	0.89 ± 0.0
20.02	295, 318	644	MS^2: 358 (8), 482 (75), 508 (100); MS^3: 332 (27), 358 (100), 372 (49)	*N^1*-feruloyl-*N^5, N^{10}*-dicaffeoylspermidine (isomer)[a,d,g]	ND	ND	ND	ND	0.18 ± 0.0	ND
20.27	295, 310	598	MS^2: 478 (46), 462 (100), 452 (46), 342 (14)	*N^1, N^5-di-p*-coumaroyl-*N^{10}*-caffeoylspermidine[a,e]	ND	ND	ND	0.22 ± 0.0	0.08 ± 0.0	ND
21.23	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine[a,e]	ND	0.13 ± 0.0	ND	0.69 ± 0.0	1.02 ± 0.0	0.42 ± 0.0
21.33	254, 268sh, 348	285	MS^2: 285 (100)	Luteolin[a,b]	ND	ND	1.00 ± 0.0	ND	ND	ND
22.22	294, 309	598	MS^2: 462 (100), 478 (39), 452 (34), 342 (14)	*N^1, N^5-di-p*-coumaroyl-*N^{10}*-caffeoylspermidine[a,d,g]	0.21 ± 0.0	0.48 ± 0.0	1.16 ± 0.0	1.40 ± 0.0	2.12 ± 0.0	0.85 ± 0.0
22.73	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine[a,e]	ND	0.16 ± 0.0	ND	1.82 ± 0.0	2.84 ± 0.0	1.68 ± 0.0
22.77	295, 310	598	MS^2: 342 (13), 452 (32), 462 (100), 478 (37)	*N^1, N^5-di-p*-coumaroyl-*N^{10}*-caffeoylspermidine (isomer)[a,e]	ND	ND	2.61 ± 0.1	ND	ND	ND

24.12	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine[a,e]	0.14 ± 0.0	0.18 ± 0.0	0.61 ± 0.0	2.13 ± 0.0	3.20 ± 0.0	0.93 ± 0.0
25.08	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine[a,e]	ND	ND	0.20 ± 0.0	0.77 ± 0.0	1.12 ± 0.1	0.34 ± 0.0
26.47	295, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	*N^1, N^5, N^{10}*-tri-*p*-coumaroylspermidine (isomer)[a,e]	0.55 ± 0.0	0.29 ± 0.0	6.28 ± 0.0	8.47 ± 0.0	15.23 ± 0.1	5.33 ± 0.0
26.92	270	785	MS^2: 665 (100), 545 (14), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine[a,l]	ND	3.34 ± 0.0	ND	ND	ND	ND
27.52	295, 308	612	MS^2: 492 (100); MS^3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine[a,l]	ND	ND	0.93 ± 0.0	0.32 ± 0.0	0.70 ± 0.0	ND
27.67	271	315	MS^2: 300 (100); MS^3: 272 (100), 255 (54), 165 (26)	Herbacetin-methyl-ether[a]	ND	ND	ND	ND	ND	0.11 ± 0.0
28.55	280, 307sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)[a,l]	0.90 ± 0.0	8.25 ± 0.1	0.76 ± 0.0	0.42 ± 0.0	0.31 ± 0.0	ND
28.77	266, 365	285	MS^2: 285 (100)	Kaempferol[a,b]	ND	ND	ND	ND	ND	0.28 ± 0.0
29.28	277, 310sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)[a,l]	ND	0.83 ± 0.1	ND	ND	ND	ND
29.57	295, 318	672	-	Polyamide derivative[a,e]	ND	ND	1.70 ± 0.0	ND	ND	ND
30.28	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)[a,l]	0.74 ± 0.0	9.11 ± 0.1	ND	0.50 ± 0.0	ND	ND
31.92	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)[a,l]	0.50 ± 0.0	6.51 ± 0.1	ND	0.47 ± 0.0	ND	0.36 ± 0.0
33.97	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)[a,l]	0.70 ± 0.0	8.49 ± 0.1	ND	0.86 ± 0.0	0.36 ± 0.1	ND
Total amount of phenolic compounds					**0.5**	**0.1**	**2.7**	**0.6**	**0.6**	**1.0**
Total amount of phenylamides					**7.9**	**38.7**	**15.9**	**20.8**	**34.2**	**11.4**

Confirmed with: [a] MS^n fragmentation; [b] Standard; References: [c] Sobral et al. (2017); [d] Negri et al. (2018); [e] El Ghouizi et al. (2020); [f] Falcão, Vale, et al. (2013); [g] Su et al. (2020); [h] Mihajlovic, Radosavljevic, Burazer, Smiljanic & Velickovic (2015); [i] Sobolev, Sy & Gloer (2008); [j] Cuyckens & Claeys (2004); [k] Barros, Dueñas, Carvalho, Ferreira & Santos-Buelga (2012); [l] Paupière et al. (2017). Values expressed as mg of each compound/g sample. ND = not detected.

The effect of *in vitro* gastrointestinal digestion on the stability of phenolic compounds and phenylamides is given in Table 6 and 7. Accordingly, after the oral phase, the bioactive compounds in the BP-A1, BP-A2 and BP-A3 samples decreased by 53%, 54% and 16%, respectively. Among the bee bread samples, the highest decrease was in BB-A3 (25%), followed by BB-A2 (17%) and BB-A1 (4%). Despite these decreases were statistically significant ($p < 0.05$), they were not very high for BP-A3, BB-A1 and BB-A2. This may be due to contact with the enzyme or short digestion time (Lucas-Gonzalez et al., 2016). These reductions were comparable to the phenolic compound results reported by Quan et al. (2020) and Lucas-Gonzalez et al. (2016) for different food matrices.

Compared with the oral phase, the phenolic content in all samples was significantly ($p < 0.05$) reduced at the end of the gastric phase. While the content of isorhamnetin-*O*-pentosyl-hexoside (*m/z* 609) in the raw BP-A1 and BP-A2 samples was 0.22 and 0.07 mg/g, respectively, its concentration in both samples decreased to a minimum of 0.05 mg/g after the gastric phase. A significant decreasing trend was also observed in the BP-A3 phenolic and phenylamide compounds when comparing to other two bee pollen samples. For the bee bread samples, a similar decrease was observed on the phenolic content at the end of the gastric phase, presenting bee bread, except for BB-A3 sample, a richer phenolic content when comparing to the bee pollen samples. As mentioned by some authors earlier (Bouayed et al., 2011; Ortega et al., 2011), the decrease in the concentration of the phenolic compounds and phenylamides in bee pollen and bee bread could be explained by the interaction with other food ingredients, causing changes in their molecular weights, solubilities, and chemical structures. Moreover, hydrolysis of the released compounds, mainly as a result of acidic pH (pH 2-3) and enzymatic activity, may have another important effect on this decrease (Kamiloglu et al., 2015).

In the intestinal phase (Table 6 and 7), there was a slight increase in the phenolic content of BP-A1 and BP-A3, while no change was observed in the BP-A2 content. On the other hand, there was a significant decrease in the phenolic content of all bee bread samples, especially in BB-A1 and BB-A2. Partial decreases and increases in the concentration of phenolic compounds after the intestinal phase could be attributed to the instability of these compounds under alkaline conditions and their possible interactions with other food components like protein, lipid or fiber, as in the gastric phase (Bouayed et al., 2011; Schulz et al., 2017). Besides, phenylamides are known to be completely

digested and absorbed in the intestine (Toro-Funes, Bosch-Fusté, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2013), and our findings show that the identified phenylamides in the samples were completely digested after the intestinal phase. In the calculation based on the total amount of phenolic compounds found in the samples, BB-A1 had the highest bioaccessibility score with a rate of 36%, followed by BB-A2 (28%) > BP-A1 (24%) > BP-A2 (14%) > BP-A3 (17%) > BB-A3 (11%) (Figure 9). After *in vitro* gastrointestinal digestion, the available quantity and diversity of compounds varied significantly ($p < 0.05$) depending on the tested sample.

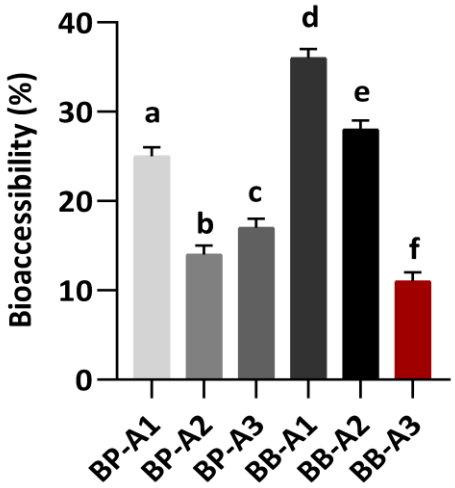

Figure 9. Bioaccessibility (%) level the total amount of phenolic compounds in bee pollen and bee bread at the end of the digestion.

The differences found in the bioavailability values are due to the diversified chemical structures found in both phenolic and phenylamide compounds which can ranged from simple molecules to highly polymerized molecules. Different conditions (enzymatic activity and/or pH changes) of gastrointestinal digestion cause various changes in the phenol structure such as hydroxylation, methylation, isoprenylation, dimerization and glycosylation, and consequently affect the stability and bioaccessibility of these compounds (G.-L. Chen et al., 2016; Lucas-Gonzalez et al., 2016; Schulz et al., 2017). Considering these findings, despite it is difficult to make a decision by comparing the stability and bioavailability of bioactive compounds in bee pollen and bee bread, it can be said that bee bread is partially a more accessible bee product compared to bee pollen.

Table 6. Phenolic and phenylamide compounds of the bee pollen samples obtained after each *in vitro* digestion phase.

Compound	Mouth			Stomach			Intestine		
	BP-A1	**BP-A2**	**BP-A3**	**BP-A1**	**BP-A2**	**BP-A3**	**BP-A1**	**BP-A2**	**BP-A3**
Quercetin-*O*-hexosyl-*O*-rutinoside	0.03 ± 0.0	ND	ND	ND	ND	ND	ND	ND	ND
Quercetin-diglucoside	ND	ND	0.03 ± 0.0	ND	ND	0.04 ± 0.0	ND	ND	0.05 ± 0.0
Methyl herbacetin-*O*-dihexoside	0.03 ± 0.0	ND	0.32 ± 0.0	ND	ND	0.17 ± 0.0	ND	ND	0.22 ± 0.0
Kaempferol-*O*-dihexoside	0.05 ± 0.0	ND	ND	ND	ND	ND	ND	ND	ND
Isorhamnetin-*O*-pentosyl-hexoside	0.12 ± 0.0	0.06 ± 0.0	ND	0.05 ± 0.0	0.05 ± 0.0	ND	0.09 ± 0.0	0.04 ± 0.0	ND
Kaempferol-3-*O*-rutinoside	0.01 ± 0.0	0.00 ± 0.0	0.03 ± 0.0	ND	ND	0.01 ± 0.0	0.01 ± 0.0	ND	0.01 ± 0.0
Quercetin-3-*O*-glucoside	0.01 ± 0.0	ND	ND	ND	ND	ND	0.01 ± 0.0	ND	ND
Quercetin-3-*O*-rhamnoside	ND	ND	0.77 ± 0.0	ND	ND	0.15 ± 0.0	ND	ND	0.14 ± 0.0
Isorhamnetin-3-*O*-malonyl glucoside	ND	ND	0.03 ± 0.0	ND	ND	0.01 ± 0.0	ND	ND	ND
Hesperetin	ND	ND	0.05 ± 0.0	ND	ND	ND	ND	ND	ND
Di-*p*-coumaroylspermidine	0.77 ± 0.0	0.03 ± 0.0	ND	ND	ND	ND	ND	ND	ND
Kaempferol-3-*O*-rhamnoside	ND	ND	0.03 ± 0.0	ND	ND	ND	ND	ND	ND
Isorhamnetin-*O*-deoxyhexoside	ND	ND	0.31 ± 0.0	ND	ND	0.02 ± 0.0	ND	ND	0.02 ± 0.0
N^1, N^5, N^{10}-*tri*-caffeoylspermidine	0.26 ± 0.0	0.04 ± 0.0	ND	ND	ND	ND	ND	ND	ND
N^1-acetyl-N^5, N^{10}-*di*-*p*-coumaroylspermidine	2.01 ± 0.1	0.35 ± 0.0	ND	ND	ND	ND	ND	ND	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine	0.14 ± 0.0	ND	0.39 ± 0.0	ND	ND	ND	ND	ND	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)	ND	ND	1.16 ± 0.0	ND	ND	ND	ND	ND	ND
N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine	0.10 ± 0.0	0.22 ± 0.0	1.17 ± 0.0	ND	ND	ND	ND	ND	ND
N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)	ND	ND	1.97 ± 0.1	ND	ND	ND	ND	ND	ND
Luteolin	ND	ND	0.99 ± 0.0	ND	ND	ND	ND	ND	ND
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine	ND	0.11 ± 0.0	0.56 ± 0.0	ND	ND	ND	ND	ND	ND
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)	0.43 ± 0.0	0.22 ± 0.0	6.31 ± 0.1	ND	ND	ND	ND	ND	ND

Tetracoumaroyl spermine	ND	ND	ND	ND	0.18 ± 0.0	ND	ND	ND	ND
Feruloyl dicoumaroyl spermidine	ND	ND	0.75 ± 0.0	ND	ND	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	ND	0.95 ± 0.0	ND	ND	ND	ND	ND	ND	ND
Diferuloyl coumarouyl spermidine	0.75 ± 0.0	ND	ND	ND	ND	ND	ND	ND	ND
Polyamide derivative	ND	ND	3.12 ± 0.2	ND	ND	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	ND	ND	ND	ND	0.36 ± 0.0	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	ND	ND	ND	ND	0.03 ± 0.0	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	ND	0.99 ± 0.0	ND	ND	0.36 ± 0.0	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	ND	ND	ND	ND	0.23 ± 0.0	ND	ND	ND	ND
The total amount of phenolic compounds	**0.2**	**0.1**	**2.6**	**0.1**	**0.0**	**0.4**	**0.1**	**0.0**	**0.4**
The total amount of phenylamides	**3.7**	**2.9**	**14.7**	**0.0**	**1.2**	**0.0**	**0.0**	**0.0**	**0.0**

Values expressed as mg of each compound/g sample. ND = not detected.

Table 7. Phenolic and phenylamide compounds of the bee bread samples obtained after each *in vitro* digestion phase.

Compound	Mouth			Stomach			Intestine		
	BB-A1	**BB-A2**	**BB-A3**	**BB-A1**	**BB-A2**	**BB-A3**	**BB-A1**	**BB-A2**	**BB-A3**
Methyl herbacetin-*O*-dihexoside	0.41 ± 0.0	0.33 ± 0.0	0.07 ± 0.0	0.22 ± 0.0	0.16 ± 0.0	0.06 ± 0.0	0.16 ± 0.0	0.12 ± 0.0	0.04 ± 0.0
Methyl herbacetin-*O*-rutinoside	0.08 ± 0.0	0.08 ± 0.0	0.01 ± 0.0	0.05 ± 0.0	0.05 ± 0.0	ND	0.04 ± 0.0	0.04 ± 0.0	ND
Isorhamnetin-*O*-pentosyl hexoside	0.05 ± 0.0	0.06 ± 0.0	0.04 ± 0.0	0.02 ± 0.0	ND	0.03 ± 0.0	0.02 ± 0.0	ND	0.04 ± 0.0
Kaempferol-3-*O*-rutinoside	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	ND	0.00 ± 0.0	0.00 ± 0.0
Quercetin-3-*O*-glucoside	ND	ND	0.01 ± 0.0	ND	ND	0.00 ± 0.0	ND	ND	ND
Quercetin-*O*-malonyl hexoside	ND	0.02 ± 0.0	0.02 ± 0.0	ND	ND	ND	ND	ND	ND
Methyl herbacetin-3-*O*-hexoside	0.02 ± 0.0	ND	ND	0.01 ± 0.0	ND	ND	ND	ND	ND
Kaempferol-*O*-hexoside	ND	0.00 ± 0.0	0.01 ± 0.0	ND	ND	ND	ND	ND	ND
Isorhamnetin-*O*-hexoside	ND	0.01 ± 0.0	ND	ND	ND	ND	ND	ND	ND
Kaempferol-*O*-malonyl hexoside	ND	0.03 ± 0.0	0.03 ± 0.0	ND	0.01 ± 0.0	0.03 ± 0.0	ND	0.01 ± 0.0	0.02 ± 0.0
N^1, N^5, N^{10}-*tri*-caffeoylspermidine	1.84 ± 0.1	4.14 ± 0.2	0.40 ± 0.0	0.07 ± 0.0	0.09 ± 0.0	ND	ND	ND	ND
N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine	ND	0.06 ± 0.0	ND	ND	ND	ND	ND	ND	ND
N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine (isomer)	ND	0.17 ± 0.0	ND	ND	ND	ND	ND	ND	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine	0.21 ± 0.0	0.13 ± 0.0	0.06 ± 0.0	ND	ND	ND	ND	ND	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)	0.50 ± 0.0	1.48 ± 0.0	0.69 ± 0.0	ND	0.03 ± 0.0	ND	ND	ND	ND
N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine	0.30 ± 0.0	0.07 ± 0.0	0.66 ± 0.0	ND	ND	ND	ND	ND	ND
N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)	1.23 ± 0.0	1.79 ± 0.1	ND	0.07 ± 0.0	ND	ND	ND	ND	ND
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine	12.25 ± 0.1	19.78 ± 0.2	7.17 ± 0.1	0.42 ± 0.0	0.18 ± 0.0	ND	ND	ND	ND
Methyl herbacetin	ND	ND	0.09 ± 0.0	ND	ND	ND	ND	ND	ND
Feruloyl dicoumaroyl spermidine	0.54 ± 0.1	0.50 ± 0.1	ND	ND	ND	ND	ND	ND	ND
Tetracoumaroyl spermine	0.59 ± 0.0	0.23 ± 0.0	ND	ND	ND	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	0.69 ± 0.0	ND	ND	ND	ND	ND	ND	ND	ND

Tetracoumaroyl spermine (isomer)	0.68 ± 0.1	ND	ND	ND	ND	ND	ND	ND	ND
Tetracoumaroyl spermine (isomer)	1.14 ± 0.1	ND	ND	ND	ND	ND	ND	ND	ND
The total amount of phenolic compounds	**0.6**	**0.6**	**0.3**	**0.3**	**0.2**	**0.1**	**0.2**	**0.2**	**0.1**
The total amount of phenylamides	**19.9**	**28.4**	**8.9**	**0.5**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**

Values expressed as mg of each compound/g sample. ND = not detected.

3.5. Antioxidant capacity

In the present study, the antioxidant capacities of undigested and digested bee pollen and bee bread samples was measured using DPPH free radicals scavenging activity and reducing power assays (Figure 8 and 9).

Concerning the evaluation of the antioxidant activity, the performance of different antioxidant assays indicates that a single one is insufficient to measure the antioxidant activities of complex phytochemicals (Apak et al., 2007). Therefore, more reliable results can be obtained by combining antioxidant assays based on different mechanisms to demonstrate more accurate antioxidant profiles of natural compounds (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). While the reducing power test measures the total antioxidant activity of hydrophilic antioxidants with ferric reduction potential (Benzie & Strain, 1996), the DPPH free radical scavenging assay measures the reaction between hydrogen atoms and peroxyl radicals in the presence of lipophilic antioxidants (Apak et al., 2007).

Among raw bee pollen samples, BP-A3 showed both the highest free radical scavenging (EC_{50}: 0.14 ± 0.00 mg/mL) and reducing power activity (499 ± 7 mg GAE/100 g), much better than BP-A1 and BP-A3. In raw bee bread samples, the behavior did not change much between samples, with BB-A2 exhibiting the highest free radical scavenging activity with a value of 0.23 ± 0.02 mg/mL and reducing power activity with 580 ± 6 mg GAE/100 g, which is in accordance with previous studies that reported that bee pollen and bee bread may have different antioxidant activity (Borycka et al., 2015; De-Melo et al., 2018; Tomás et al., 2017).

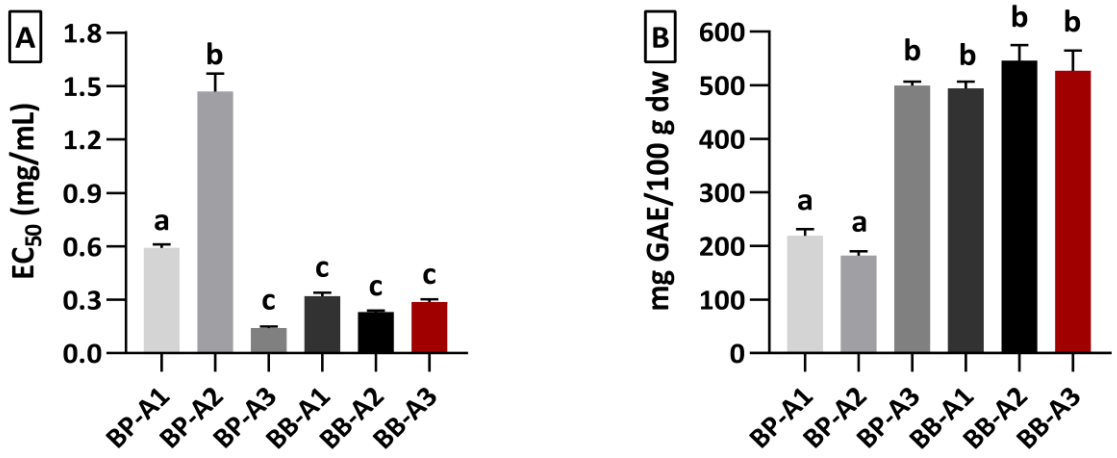

Figure 10. Antioxidant capacities of undigested (raw) bee pollen and bee bread samples: (A) DPPH free radical scavenging and (B) reducing power activity.

At the end of *in vitro* gastrointestinal digestion, a decrease of 35-85% in free radicals scavenging capacity and of 47-76% in reducing power occurred for bee pollen samples, while in bee bread samples, there was a decrease of 69-74% in free radicals scavenging capacity and of 33-50% in reducing power activity, Figure 9. In addition, all bee pollen samples showed a steadily decreasing trend in terms of DPPH scavenging activity after each digestion phase. The same situation was observed in reducing power activity, except in the intestinal phase for BP-A3. As expected, the BP-A3, which has the highest antioxidant activity in the undigested samples, also showed the highest activity at the end of digestion, for both tests. These differences in bee pollen samples may be related to the high content released from samples and slowing down in the intestinal phase or the degradation of the released compounds under conditions in the intestinal phase (Ng & See, 2019; Schulz et al., 2017).

All bee bread samples showed a higher antioxidant capacity at the end of the intestinal phase compared to the gastric phase. Several studies reported the changes, amounts and antioxidant activities of the phenolic contents of different food matrices during digestion, similarly, they emphasized that antioxidant activity was higher in the intestinal phase compared to the gastric phase (Kamiloglu et al., 2015; Quan et al., 2018; Yesiltas et al., 2014). All the tested samples showed different antioxidant activity at each phase of digestion. Because of the amount and structure of phenolic compounds in the samples, their interactions with macronutrients or the presence of anthocyanins may cause them to exhibit different antioxidant activity (Ng & See, 2019). A comparison between bee pollen and bee bread revealed that bee bread showed higher antioxidant activity at the end of digestion than bee pollen with two different antioxidant assays.

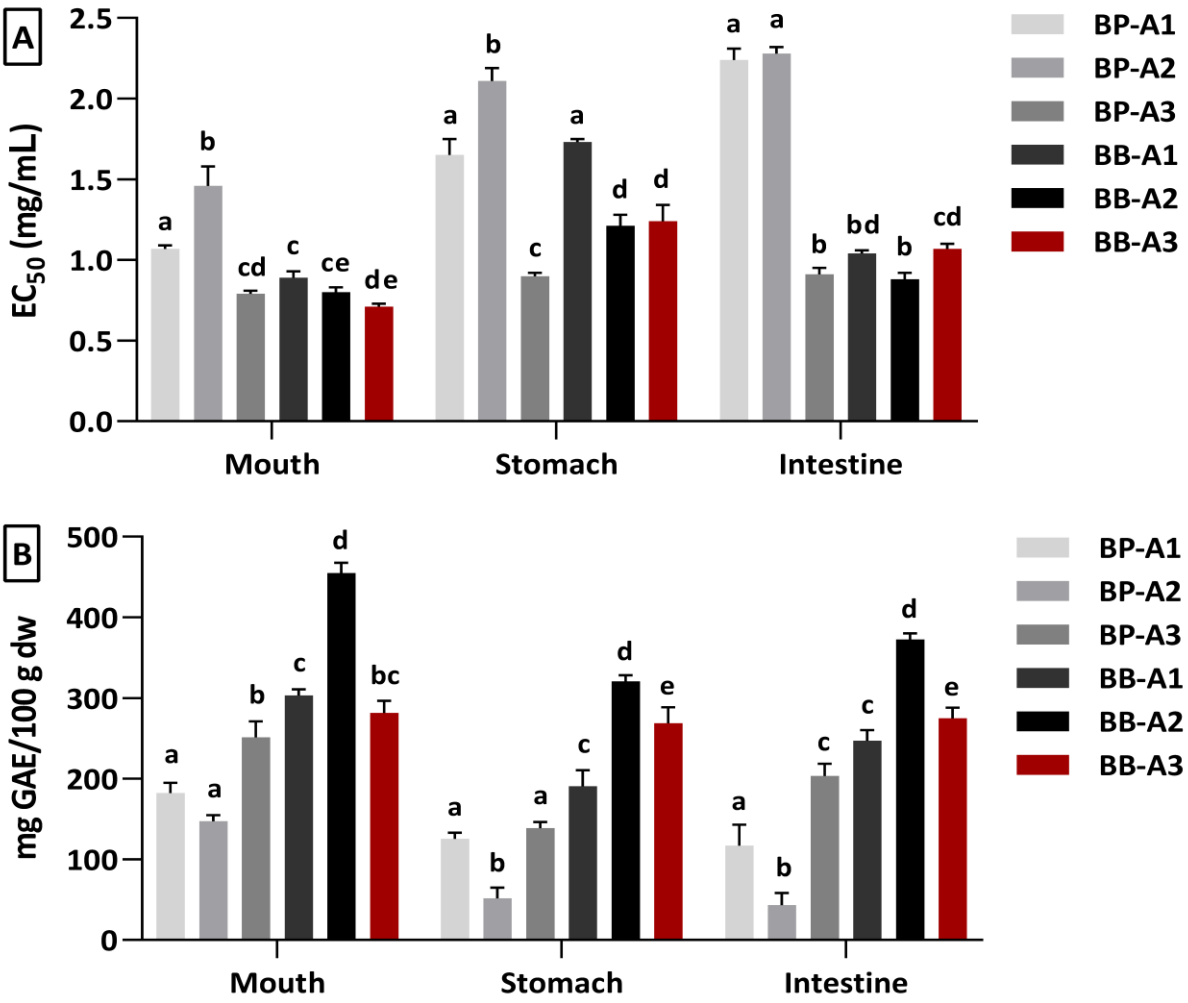

Figure 11. Antioxidant capacities of bee pollen and bee bread samples after each *in vitro* digestion phase: (A) DPPH free radical scavenging and (B) reducing power activity.

The correlation between bioactive compounds and antioxidant capacity in bee pollen and bee bread was evaluated and the results are given in Table 7. The DPPH value showed significant and very strong positive correlation with both TPC ($r = 0.799$; $p < 0.01$) and TFC ($r = 0.784$; $p < 0.01$). The reducing power value was positively correlated strongly with TPC ($r = 0.743$; $p < 0.01$), while it was moderately correlated with TFC ($r = 0.562$; $p < 0.01$). The moderate correlation of reducing power with TFC compared to TPC could be attributed to the effect of digestion on the structure of flavonoids, and this is clearly seen in Figure 7. Besides, these results are supported by the findings given above, where samples with high TPC and TFC generally exhibit higher antioxidant activity. Correlation analysis indicates that the antioxidant compounds in bee pollen and bee bread are not only potent radical scavengers but also good reducing agents.

Table 8. Correlation analysis between phenolic content and antioxidant capacity.

Assays	r	Confidence interval of r	Significance
Between phenolic content and antioxidant capacity			
TPC – DPPH	0,799	0.584 to 0.909	**
TPC – Reducing power	0.743	0.484 to 0.881	**
TFC – DPPH	0.784	0.557 to 0.902	**
TFC – Reducing power	0.562	0.419 to 0.862	**
Between phenolic and flavonoid			
TPC – TFC	0.873	0.724 to 0.943	**
Between DPPH and reducing power			
DPPH – Reducing power	0.839	0.658 to 0.928	**

Twenty-four paired average samples from each test were used in the comparison. r value represents the Pearson's linear correlation value. The level of significance was expressed as **$p < 0.01$.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. Overview and conclusions

The main objective of this thesis was to comparatively determine the physicochemical parameters and the bioavailability properties of bee pollen and bee bread using an *in vitro* static gastrointestinal digestion model. In order to successfully fulfill the aims of the work, several aspects and tests were applied. Specifically, the work involved the adjustment of bee pollen and bee bread according to the *in vitro* gastrointestinal digestion model and successful implementation of it; possible factors affecting the bioavailability of bioactive compounds in each digestive phase; and the resulting changes in their antioxidant capacity.

The palynological analysis showed that pollen types from Plantaginaceae, Asteraceae, and Fabaceae plant families were dominant in the bee pollen samples. For bee bread samples, the presence of dominant taxon was not so evident, nevertheless the major contribution was from Fabaceae, Fagaceae, Campanulaceae and Rosaceae. Depending on this, the diversity of pollen grains caused both bee pollen and bee bread to have different physicochemical properties: the average lipid content of the bee pollen samples was 1.4 times higher than bee bread but had a lower content in terms of protein and total carbohydrates. Similar to these compositional properties, bee pollen and bee bread differed significantly in terms of total phenolic and flavonoid content, accordingly to the botanical origin. Besides, quercetin, kaempferol, and isorhamnetin were determined as major phenolics in bee pollen and bee bread, while polyamines spermidines and their derivatives were determined as compounds found in high concentrations in both bee products.

The presence of the monosaccharides fructose and glucose was significant in both bee products, while sucrose was only observed in bee pollen. The presence of other sugars was also registered, but at lower concentrations. A particular behavior was found when comparing the fructose/glucose ratio, which was significantly higher for bee bread samples. This may be result from the addition of nectar during the bee bread processing, but also due to the use of glucose in the metabolic processes of the lactic acid bacteria present in bee bread.

The amount of total phenolic and flavonoid content was highly affected (tended to decrease) by the GIT, which was reflected in the bioavailability scores of the samples. This decrease was particularly more dramatic after the gastric phase, with an average reduction of 54% and 53% in bee pollen, and 67% and 52% in bee bread, for TPC and TFC, respectively. Contrarily, the phenolic content slightly increased in the intestinal

phase, particularly for bee bread samples, what shows that the acidic ambient and enzyme are important factors on the bioavailability of phenolic compounds. Comparatively, after completion of all the digestive process, bee bread samples revealed higher bioaccessibility levels for both TPC and TFC with 38% and 35%, respectively, which can be explained by the partial digestion of the multi-layered structure of pollen grains by bacterial enzymes throughout the fermentation process of bee bread.

The reduction trend observed in the concentrations of bioactive compounds in bee pollen and bee bread was also reflected in their antioxidant activities. The gastrointestinal digestion conduct to a decrease of 35-85% in free radicals scavenging capacity and of 47-76% in reducing power for bee pollen samples, while in bee bread samples, there was a decrease of 69-74% in free radicals scavenging capacity and of 33-50% in reducing power activity.

Considering the obtained results, it was shown that the phenolic content in both raw products does not reflect the real amount absorbed in the intestinal lumen, nevertheless, it was evident that bee bread has both a richer phenolic content and bioavailability level as well as a better nutritional value when compared to bee pollen.
Finally, it is important to mention that during the execution of this work, and with its outputs, it was possible to prepare one review paper on the nutrition value, digestibility and bioavailability of the dietary phytochemicals present in the bee pollen and bee bread, which under review on an indexed journal, and a second one, focused on the experimental results of the digestion process, is now under the submission process. A third paper focused on the comparison of nutritional properties of both pollen and bee bread is also in preparation. The abstract of these manuscripts can be seen in the appendices.

4.2. Future perspectives

Even though the main goals have been achieved, some studies are needed to better understand the bioactivity fates in the body and modifications that bee pollen and bee bread undergo in the gastrointestinal tract. Hence, several recommendations for improvement of the current work and guidelines for future work in this research area can be developed:

– Bee pollen and bee bread have been shown to be rich sources of both macronutrients and phytochemicals, however, both bee pollen lack a certain standardization, so future research should focus on developing the standardization depending on the botanical origin of pollen;

- Despite the results from *in vitro* gastrointestinal digestion cannot directly predict the results from *in vivo* studies, they provide important baseline information for further studies. To support the findings of this study, implementing a model involving a colon microbial digestion with cellular models like Caco-2 or conducting direct *in vivo* studies;
- Finally, conducting studies on protecting the phytochemicals in bee pollen and bee bread, which are thought to have important biological activities, against the harsh conditions of the GIT with edible coatings.

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APPENDICES

1. The LC-MS/MS^n results of samples

The LC-MS/MS^n results of raw and digested samples of bee pollen and bread are given in Table A1/Figure A1 – Table A24/Figure A24 with the chromatograms for each sample.

Table A1. Phenolic compounds and phenylamides profile of the raw BP-A1 sample.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	6.58	255, 349	771	MS^2: 609 (100); MS^3: 301 (100)	Quercetin-*O*-hexosyl-*O*-rutinoside
2	8.37	272, 326sh, 353sh	639	MS^2: 315(100), 477(70); MS^3: 300, 301, 287	Methyl herbacetin-*O*-dihexoside
3	9.94	265, 348	609	MS^2: 285 (100), 429 (49)	Kaempferol-*O*-dihexoside
4	11.69	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
5	12.09	265, 348	593	MS^2: 284 (100), 285 (73), 429 (93)	Kaempferol-*O*-rutinoside
6	12.62	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside
7	15.39	299, 308	436	MS^2: 316 (100)	di-*p*-coumaroylspermidine
8	16.77	295, 318	630	MS^2: 468 (100), 494 (85), 358 (8), 332 (6); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
9	18.18	299, 308	478	MS^2: 358 (100), 332 (12), 145 (5)	N^1-acetyl-N^5, N^{10}-*di*-*p*-coumaroylspermidine
10	18.86	296, 314	614	MS^2: 478 (100), 479 (24), 468 (20); MS^3: 342 (100), 332 (75)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
11	22.22	294, 309	598	MS^2: 462 (100), 478 (39), 452 (34), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
12	24.00	292, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer)
13	26.47	295, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer)
14	28.57	216, 275	785	MS^2: 545 (14), 639 (13), 665 (100); MS^3: 545 (100), 546 (33)	Tetracoumaroyl spermine
15	30.28	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
16	31.92	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
17	33.97	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

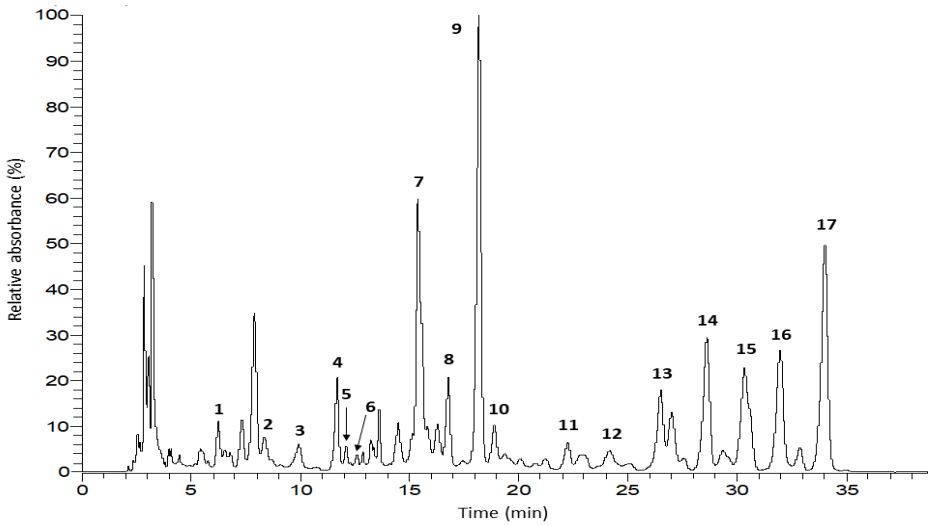

Figure A1. Chromatographic profile of raw BP-A1 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A2. Phenolic compounds and phenylamides profile of the raw BP-A2 sample.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.90	201, 252, 341sh	830	–	ND
2	11.69	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	12.10	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
4	12.29	289, 352	668	MS^2: 623 (100), 379 (40), 574 (9)	ND
5	13.62	216, 355	668	MS^2: 379 (100), 375 (43), 285 (16)	ND
6	15.39	299, 309	436	MS^2: 316 (100)	Di-*p*-coumaroylspermidine
7	16.76	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
8	18.17	299, 306	478	MS^2: 358 (100), 332 (12), 145 (5)	N^1-acetyl-N^5, N^{10}-*di-p*-coumaroylspermidine
9	21.23	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine
10	22.22	294, 309	598	MS^2: 462 (100), 478 (39), 452 (34), 342 (14)	N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine
11	22.75	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine
12	24.13	292, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)
13	26.47	295, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)
14	26.96	270	785	MS^2: 665 (100), 545 (14), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine
15	28.57	280, 307sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
16	29.28	277, 310sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
17	30.28	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
18	31.92	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
19	33.97	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

ND = not detected.

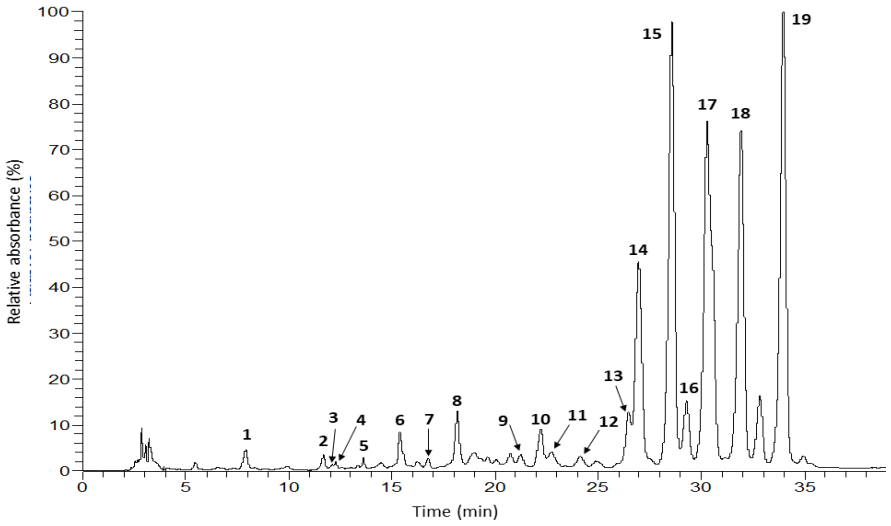

Figure A2. Chromatographic profile of raw BP-A2 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A3. Phenolic compounds and phenylamides profile of the raw BP-A3 sample.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.48	257, 353	625	MS^2: 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin-diglucoside
2	8.45	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11)	Methyl herbacetin-*O*-dihexoside
3	12.09	267, 347	593	MS^2 : 284 (100), 285 (69)	Kaempferol-3-*O*-rutinoside
4	14.18	254, 347	447	MS^2: 301 (100)	Quercetin-3-*O*-rhamnoside
5	14.44	255, 354	563	MS^2: 519 (100); MS^3: 315 (100)	Isorhamnetin-3-*O*-malonyl glucoside
6	14.73	277, 311	301	MS^2: 283 (100), 286 (40)	Hesperetin
7	16.18	264, 341	431	MS^2: 285 (100)	Kaempferol-3-*O*-rhamnoside
8	16.64	255, 354	461	MS^2: 314 (100), 315 (77), 299 (39)	Isorhamnetin-*O*-deoxyhexoside
9	18.09	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
10	19.33	295, 311	614	MS^2: 494 (25), 478 (100), 452 (71), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
11	21.33	254, 268sh, 348	285	MS^2: 285 (100)	Luteolin
12	22.19	295, 310	598	MS^2: 342 (13), 452 (32), 462 (100), 478 (37)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
13	22.76	295, 310	598	MS^2: 342 (13), 452 (32), 462 (100), 478 (37)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)
14	24.11	295, 310	582	MS^2: 462 (100), 436 (6), 342 (4)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
15	25.09	295, 310	582	MS^2: 462 (100), 436 (10), 342 (8)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)
16	26.45	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)
17	27.52	295, 308	612	MS^2: 492 (100); MS^3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine
18	28.55	295, 314	642	MS^2: 466 (16), 492 (78), 506 (57), 522 (100); MS^3: 479 (100)	Diferuloyl coumarouyl spermidine
19	29.57	295, 318	672	–	Polyamide derivative

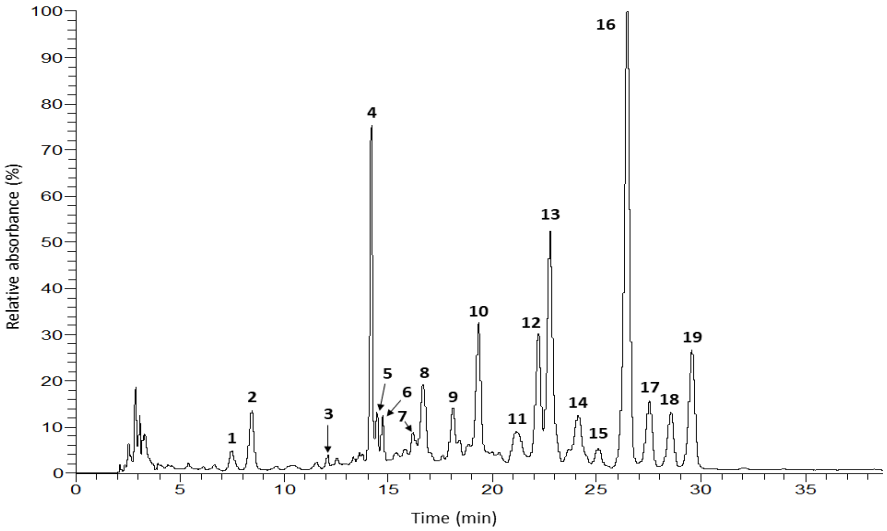

Figure A3. Chromatographic profile of raw BP-A3 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A4. Phenolic compounds and phenylamides profile of the raw BB-A1 sample.

Peak	t_R (min)	λ_{max} (nm)	[M-H]$^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.44	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.69	272, 326sh, 353sh	623	MS^2: 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.67	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.08	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100)	Kaempferol-3-*O*-rutinoside
5	13.61	270	477	MS^2: 315 (100), 462 (42), 300 (14); MS^3: 300 (100)	Methyl herbacetin-3-*O*-hexoside
6	15.76	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-caffeoylspermidine
7	16.75	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-caffeoylspermidine
8	18.14	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	*N*^{1}-*p*-coumaroyl-*N*^{5}, *N*^{10}-dicaffeoylspermidine
9	18.74	295, 311	614	MS^2: 478 (100), 468 (20), 452 (68), 342(5)	*N*^{1}-*p*-coumaroyl-*N*^{5}, *N*^{10}-dicaffeoylspermidine (isomer)
10	19.34	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	*N*^{1}-*p*-coumaroyl-*N*^{5}, *N*^{10}-dicaffeoylspermidine (isomer)
11	20.27	295, 310	598	MS^2: 478 (46), 462 (100), 452 (46), 342 (14)	*N*^{1}, *N*^{5}-*di*-*p*-coumaroyl-*N*^{10}-caffeoylspermidine
12	21.20	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-*p*-coumaroylspermidine
13	22.19	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	*N*^{1}, *N*^{5}-*di*-*p*-coumaroyl-*N*^{10}-caffeoylspermidine (isomer)
14	22.75	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-*p*-coumaroylspermidine
15	24.12	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-*p*-coumaroylspermidine
16	25.08	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-*p*-coumaroylspermidine
17	26.46	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	*N*^{1}, *N*^{5}, *N*^{10}-*tri*-*p*-coumaroylspermidine
18	27.58	295, 308	612	MS2: 492 (100); MS3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine
19	28.57	280, 307sh	785	MS^2: 665 (100), 545 (27), 519 (11),520; MS^3: 545 (100)	Tetracoumaroyl spermine
20	30.26	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
21	31.92	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
22	33.96	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

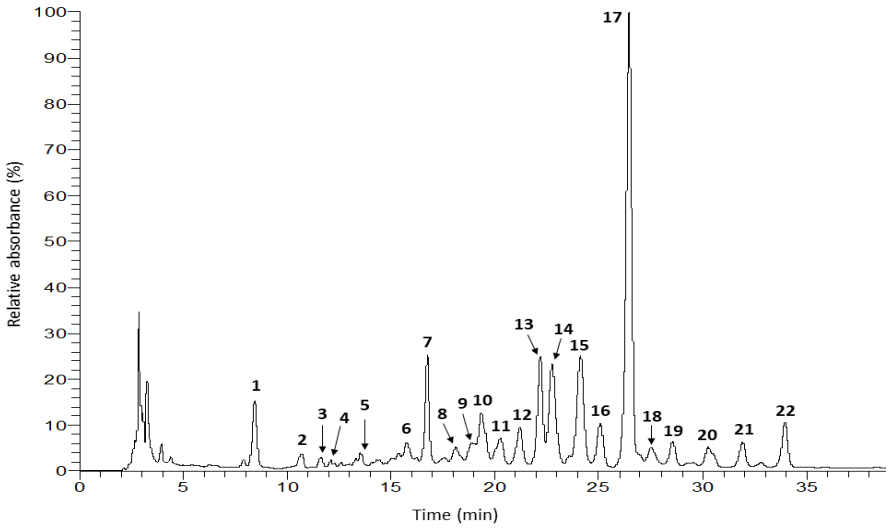

Figure A4. Chromatographic profile of raw BB-A1 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A5. Phenolic compounds and phenylamides profile of the raw BB-A2 sample.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.43	272, 326sh, 353	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.68	272, 326sh, 353	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.60	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.10	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	13.31	256, 353	549	MS^2: 505 (100); MS^3: 301 (100), 300 (28), 463 (26)	Quercetin-*O*-malonyl hexoside
6	14.06	265, 347	447	MS^2: 285 (100), 284 (80)	Kaempferol-*O*-hexoside
7	14.31	254, 355	477	MS^2: 314 (100), 315 (45)	Isorhamnetin-*O*-hexoside
8	14.99	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside
9	15.76	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
10	16.75	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
11	17.57	293, 314	644	MS^2: 358 (11), 482 (11), 508 (100); MS^3: 332 (27), 358 (100), 372 (49)	N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine
12	18.06	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
13	18.74	295, 311	614	MS^2: 478 (100), 468 (20), 452 (68), 342(5)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
14	19.33	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
15	20.02	295, 318	644	MS^2: 358 (8), 482 (75), 508 (100); MS^3: 332 (27), 358 (100), 372 (49)	N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
16	20.29	295, 310	598	MS^2: 478 (46), 462 (100), 452 (46), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
17	21.20	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
18	22.19	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)
19	22.76	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
20	24.12	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
21	25.07	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
22	26.47	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
23	27.51	295, 308	612	MS2: 492 (100); MS3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine

24	28.56		785	MS^2: 665 (100), 666 (27), 545 (14); MS^3: 545 (100), 546 (20)	Tetracoumaroyl spermine
25	33.95	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

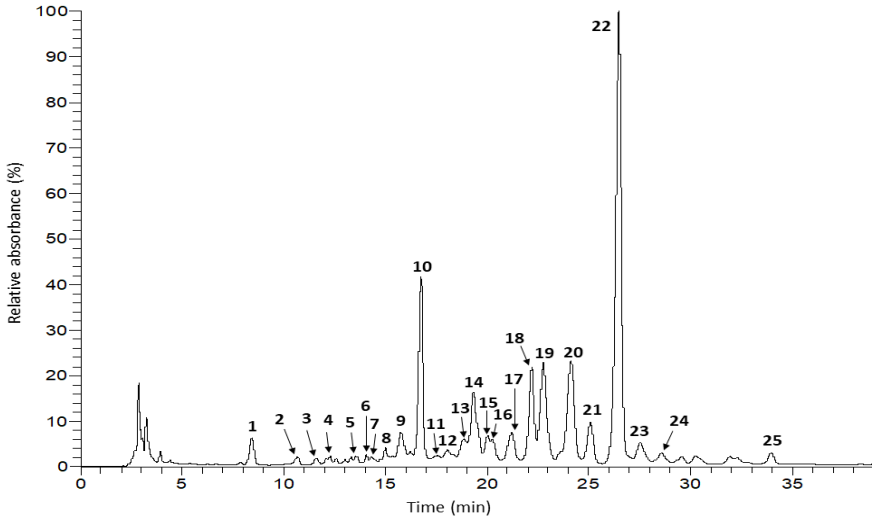

Figure A5. Chromatographic profile of raw BB-A2 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A6. Phenolic compounds and phenylamides profile of the raw BB-A3 sample.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.43	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.69	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.61	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.11	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	12.58	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside
6	13.31	256, 353	549	MS^2: 505 (100); MS^3: 301 (100), 300 (28), 463 (26)	Quercetin-*O*-malonyl hexoside
7	14.06	265, 347	447	MS^2: 285 (100), 284 (80)	Kaempferol-*O*-hexoside
8	14.31	254, 355	477	MS^2: 314 (100), 315 (45)	Isorhamnetin-*O*-hexoside
9	14.98	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside
10	16.74	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
11	18.05	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
12	19.31	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
13	21.18	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
14	22.18	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
15	22.75	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
16	24.10	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
17	25.04	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
18	26.45	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
19	27.67	271	315	MS^2: 300 (100); MS^3: 272 (100), 255 (54), 165 (26)	Methyl herbacetin
20	28.77	266, 365	285	MS^2: 285 (100)	Kaempferol
21	31.93	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

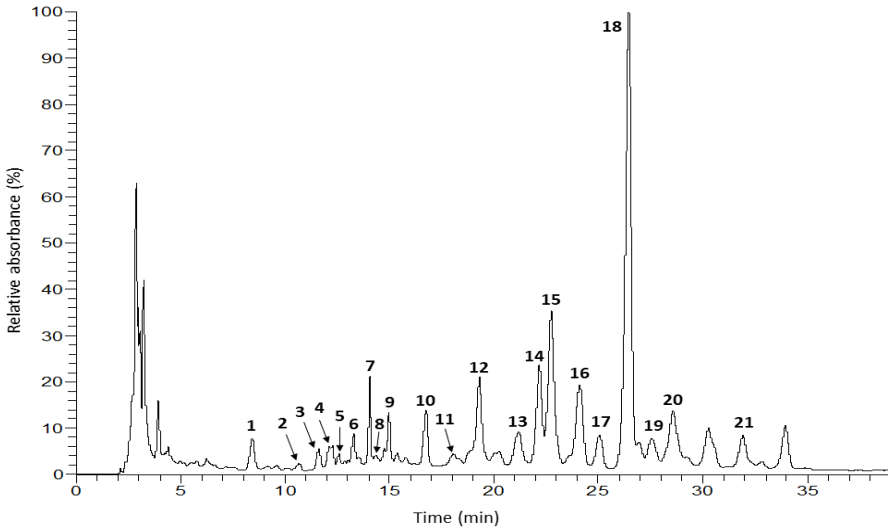

Figure A6. Chromatographic profile of raw BB-A3 sample obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A7. Phenolic compounds and phenylamides profile of the digested BP-A1 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	6.42	255, 349	771	MS^2: 609 (100); MS^3: 301 (100)	Quercetin-*O*-hexosyl-*O*-rutinoside
2	7.74	252, 267, 346	831	MS^2: 785 (100)	ND
3	8.21	272, 326sh, 353sh	639	MS^2: 315(100), 477(70); MS^3: 300 (100), 301 (51)	Methyl herbacetin-*O*-dihexoside
4	9.94	265, 348	609	MS^2: 285 (100), 429 (49)	Kaempferol-*O*-dihexoside
5	11.63	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
6	12.07	265, 348	593	MS^2: 284 (100), 285 (73), 429 (93)	Kaempferol-*O*-rutinoside
7	12.56	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside
8	13.59	253, 356	669	MS^2: 379 (100), 623 (6), 285 (14)	ND
9	15.16	299, 308	436	MS^2: 316 (100)	di-*p*-coumaroylspermidine
10	15.59	299, 308	436	MS^2: 316 (100)	di-*p*-coumaroylspermidine
11	16.81	295, 318	630	MS^2: 468 (100), 494 (85), 358 (8), 332 (6); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
12	18.28	299, 308	478	MS^2: 358 (100), 332 (12), 145 (5)	N^1-acetyl-*N5*, N^{10}-*di-p*-coumaroylspermidine
13	18.96	296, 314	614	MS^2: 478 (100), 479 (24), 468 (20), 452 (7); MS^3: 342 (100), 332 (75)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
14	22.38	294, 309	598	MS^2: 462 (100), 478 (39), 452 (34), 342 (14)	N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine
15	24.29	292, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer)
16	26.70	295, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine (isomer)

ND = not detected.

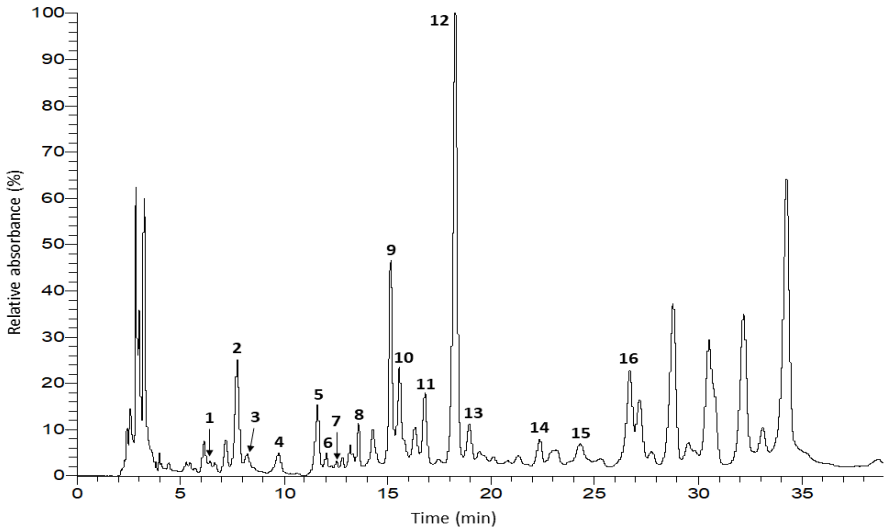

Figure A7. Chromatographic profile of digested BP-A1 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A8. Phenolic compounds and phenylamides profile of the digested BP-A2 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.89	201, 252	830	–	ND
2	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	12.14	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
4	12.29	289, 352	623	–	ND
5	15.64	299, 309	436	MS^2: 316 (100)	di-*p*-coumaroylspermidine
6	17.07	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
7	18.31	299, 306	478	MS^2: 358 (100), 332 (12), 145 (5)	N^1-acetyl-N^5, N^{10}-*di-p*-coumaroylspermidine
8	21.37	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine
9	22.37	294, 309	598	MS^2: 462 (100), 478 (39), 452 (34), 342 (14)	N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine
10	22.95	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine
11	24.42	292, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)
12	26.75	295, 305	582	MS^2: 342 (100), 436 (9), 462 (100)	N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)
13	27.19	270	785	MS^2: 399; MS^3: 399	ND
14	28.57	280, 307sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
15	30.54	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
16	32.22	293	830	–	ND
17	34.29	299, 308	831	–	ND

ND = not detected.

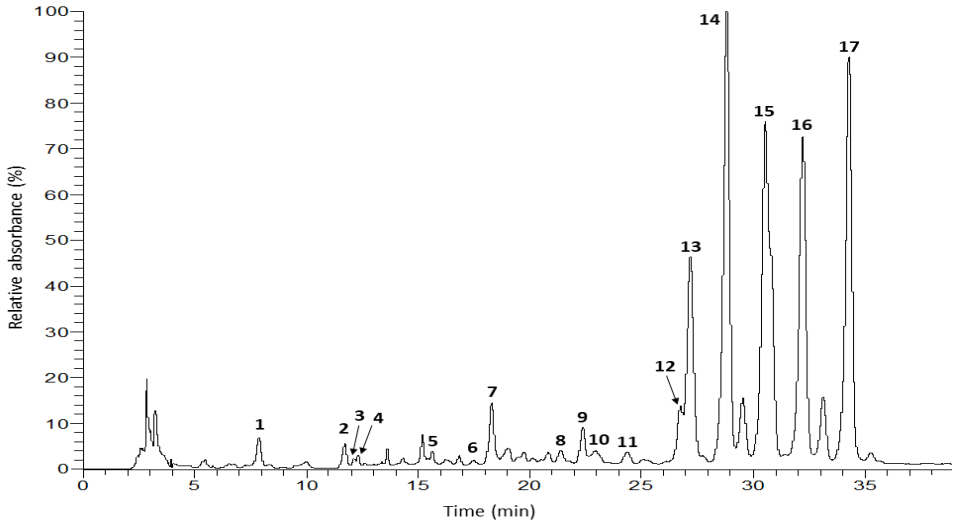

Figure A8. Chromatographic profile of digested BP-A2 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A9. Phenolic compounds and phenylamides profile of the digested BP-A3 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.54	257, 353	625	MS^2: 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin-diglucoside
2	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	12.15	267, 347	593	MS^2 : 284 (100), 285 (69)	Kaempferol-3-*O*-rutinoside
4	14.22	254, 347	447	MS^2: 301 (100)	Quercetin-3-*O*-rhamnoside
5	14.48	255, 354	563	MS^2: 519 (100); MS^3: 315 (100)	Isorhamnetin-3-*O*-malonyl glucoside
6	14.82	277, 311	301	MS^2: 283 (100), 286 (40)	Hesperetin
7	16.22	264, 341	431	MS^2: 285 (100)	Kaempferol-3-*O*-rhamnoside
8	16.70	255, 354	461	MS^2: 314 (100), 315 (77), 299 (39)	Isorhamnetin-*O*-deoxyhexoside
9	18.23	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
10	19.47	295, 311	614	MS^2: 494 (25), 478 (100), 452 (71), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
11	21.30	254, 268sh, 348	285	MS^2: 285 (100)	Luteolin
12	22.39	295, 310	598	MS^2: 342 (13), 452 (32), 462 (100), 478 (37)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
13	22.97	295, 310	598	MS^2: 342 (13), 452 (32), 462 (100), 478 (37)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)
14	24.33	295, 310	582	MS^2: 462 (100), 436 (6), 342 (4)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
15	25.32	295, 310	582	MS^2: 462 (100), 436 (10), 342 (8)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)
16	26.74	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)
17	27.80	295, 308	612	MS^2: 492 (100); MS^3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine
18	28.86	295, 314	642	MS^2: 466 (16), 492 (78), 506 (57), 522 (100); MS^3: 479 (100)	Diferuloyl coumarouyl spermidine
19	29.88	295, 318	672	–	Polyamide derivative

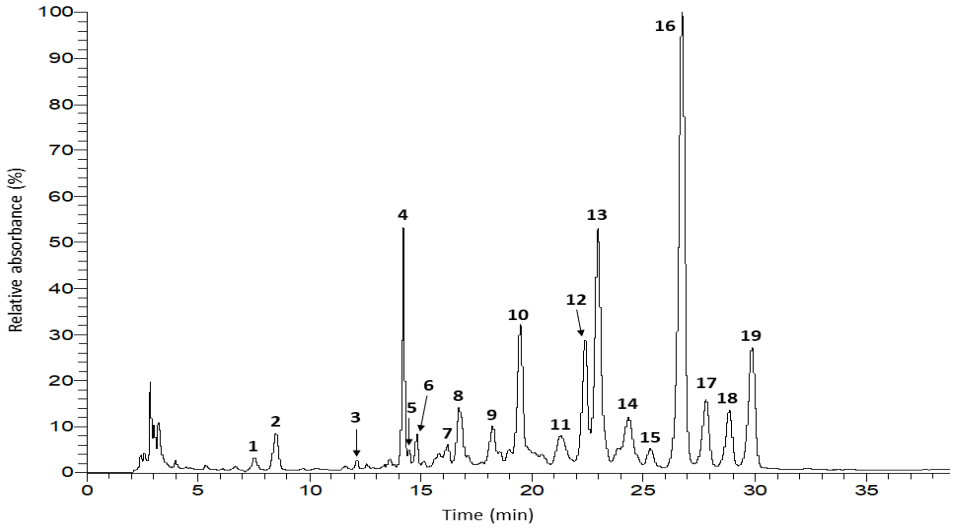

Figure A9. Chromatographic profile of digested BP-A3 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A10. Phenolic compounds and phenylamides profile of the digested BB-A1 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.49	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.77	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.14	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	13.63	270	477	MS^2: 315 (100), 462 (42), 300 (14); MS^3: 300 (100)	Methyl herbacetin-3-*O*-hexoside
6	15.82	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	*N^1, N^5, N^{10}-tri*-caffeoylspermidine
7	16.84	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	*N^1, N^5, N^{10}-tri*-caffeoylspermidine
8	18.29	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine
9	19.00	295, 311	614	MS^2: 478 (100), 468 (20), 452 (68), 342(5)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine (isomer)
10	19.48	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	*N^1-p*-coumaroyl-*N^5, N^{10}*-dicaffeoylspermidine (isomer)
11	20.42	295, 310	598	MS^2: 478 (46), 462 (100), 452 (46), 342 (14)	*N^1, N^5-di-p*-coumaroyl-*N^{10}*-caffeoylspermidine
12	21.39	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine
13	22.37	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	*N^1, N^5-di-p*-coumaroyl-*N^{10}*-caffeoylspermidine (isomer)
14	22.96	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine
15	24.35	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine
16	25.32	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine
17	26.74	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine
18	27.93	295, 308	612	MS2: 492 (100); MS3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine

19	28.83	280, 307sh	785	MS^2: 665 (100), 545 (27), 519 (11),520; MS^3: 545 (100)	Tetracoumaroyl spermine
20	30.26	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
21	32.23	293, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
22	34.31	299, 310	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)

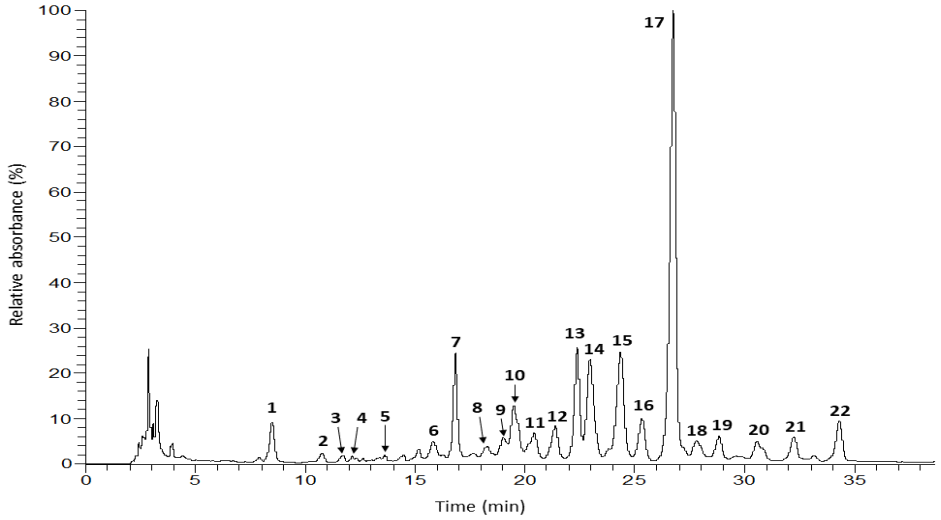

Figure A10. Chromatographic profile of digested BB-A1 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A11. Phenolic compounds and phenylamides profile of the digested BB-A2 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.77	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.72	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.15	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	13.37	256, 353	549	MS^2: 505 (100); MS^3: 301 (100), 300 (28), 463 (26)	Quercetin-*O*-malonyl hexoside
6	14.09	265, 347	447	MS^2: 285 (100), 284 (80)	Kaempferol-*O*-hexoside
7	14.34	254, 355	477	MS^2: 314 (100), 315 (45)	Isorhamnetin-*O*-hexoside
8	15.04	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside
9	15.83	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
10	16.84	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
11	17.68	293, 314	644	MS^2: 358 (11), 482 (11), 508 (100); MS^3: 332 (27), 358 (100), 372 (49)	N^1-feruloyl-N^5, N^{10}-dicaffeoylspermidine
12	18.18	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine
13	19.48	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
14	20.16	295, 318	644	MS^2: 358 (8), 482 (75), 508 (100);	N^1-feruloyl-N^5, N^{10}-

				MS^3: 332 (27), 358 (100), 372 (49)	dicaffeoylspermidine (isomer)
15	20.42	295, 310	598	MS^2: 478 (46), 462 (100), 452 (46), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
16	21.39	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
17	22.38	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)
18	22.97	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
19	24.35	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
20	25.33	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
21	26.74	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
22	27.84	295, 308	612	MS2: 492 (100); MS3: 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine
23	28.83	–	785	MS^2: 665 (100), 666 (27), 545 (14); MS^3: 545 (100), 546 (20)	Tetracoumaroyl spermine

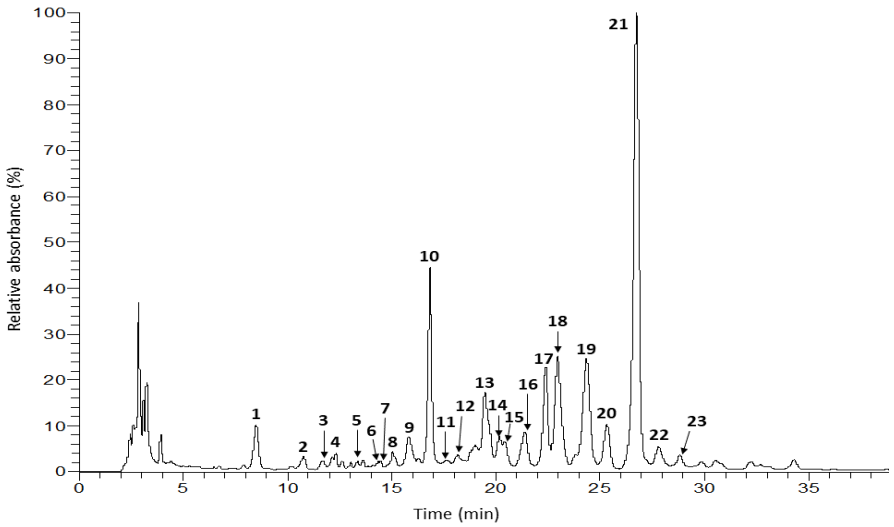

Figure A11. Chromatographic profile of digested BB-A2 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A12. Phenolic compounds and phenylamides profile of the digested BB-A3 sample (oral phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.78	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.16	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	12.62	256, 354	463	MS^2: 301 (100)	Quercetin-3-O-glucoside
6	13.36	256, 353	549	MS^2: 505 (100); MS^3: 301 (100), 300 (28), 463 (26)	Quercetin-*O*-malonyl hexoside
7	14.05	265, 347	447	MS^2: 285 (100), 284 (80)	Kaempferol-*O*-hexoside
8	15.02	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside
9	15.78	295, 315	630	MS^2: 468 (100), 494 (84), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
10	16.83	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
11	18.16	295, 311	614	MS^2: 494 (25), 478 (100), 452 (69), 358 (20)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine

12	18.79	295, 311	614	MS^2: 478 (100), 468 (20), 452 (68), 342(5)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
13	19.45	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
14	21.34	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
15	22.37	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine
16	22.95	295, 310	582	MS^2: 462 (100), 436 (9), 342(7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
17	24.33	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
18	25.24	295, 310	582	MS^2: 462 (100), 436 (9), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
19	26.72	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
20	27.88	271	315	MS^2: 300 (100); MS^3: 272 (100), 255 (54), 165 (26)	Methyl herbacetin
21	30.53	253, 370	315	MS^2: 300 (100)	ND

ND = not detected.

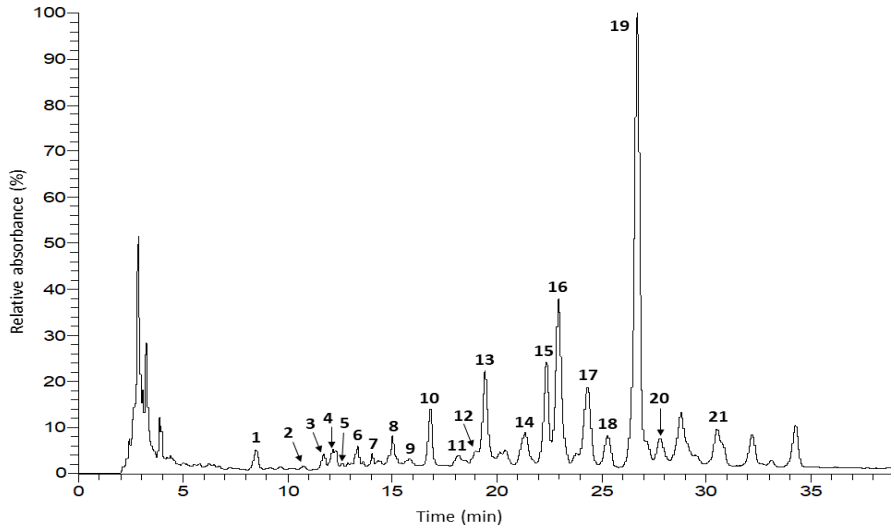

Figure A12. Chromatographic profile of digested BB-A3 sample (oral phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A13. Phenolic compounds profile of the digested BP-A1 sample (gastric phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.91	252, 267, 346	831	785	ND
2	11.74	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	13.64	253, 356	669	MS^2: 379 (100), 623 (6), 285 (14)	ND

ND = not detected.

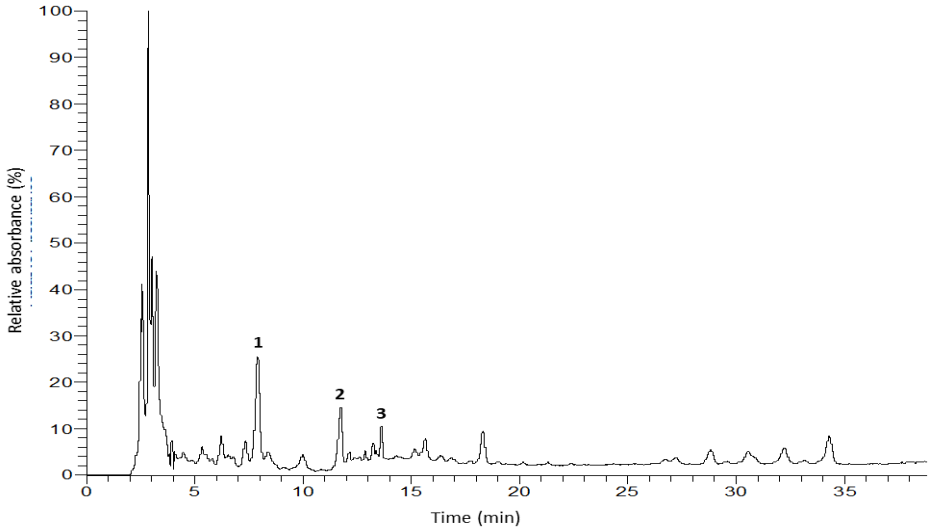

Figure A13. Chromatographic profile of digested BP-A1 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A14. Phenolic compounds and phenylamides profile of the digested BP-A2 sample (gastric phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.91	201, 252, 341	830	–	ND
2	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	12.33	289, 352	623	–	ND
4	13.63	217	668	MS^2: 379, 380, 285	ND
5	27.20	270	785	MS^2: 665 (100), 545 (14), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine
6	28.82	280, 307sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
7	29.62	277, 310sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
8	30.52	289, 306sh	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
9	32.23	293	785	MS^2: 665 (100), 545 (13), 639 (13); MS^3: 545 (100)	Tetracoumaroyl spermine (isomer)
10	34.30	299, 308	831	–	ND

ND = not detected.

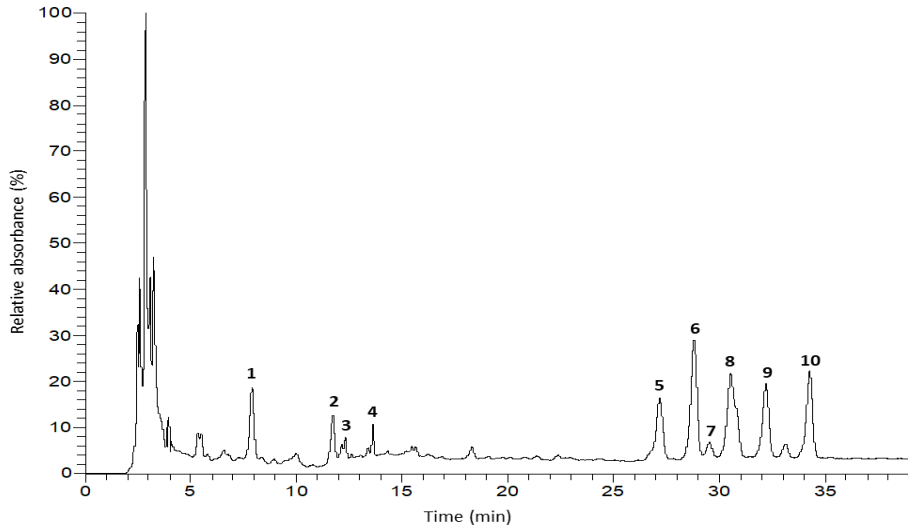

Figure A14. Chromatographic profile of digested BP-A2 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A15. Phenolic compounds profile of the digested BP-A3 sample (gastric phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.54	257, 353	625	MS^2: 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin-diglucoside
2	8.51	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	12.09	267, 347	593	MS^2 : 284 (100), 285 (69)	Kaempferol-3-*O*-rutinoside
4	13.64	218	669	MS^2: 379, 575, 576, 285, 380	ND
5	14.18	254, 347	447	MS^2: 301 (100)	Quercetin-3-*O*-rhamnoside
6	14.44	255, 354	563	MS^2: 519 (100); MS^3: 315 (100)	Isorhamnetin-3-*O*-malonyl glucoside
7	16.71	255, 354	461	MS^2: 314 (100), 315 (77), 299 (39)	Isorhamnetin-*O*-deoxyhexoside

ND = not detected.

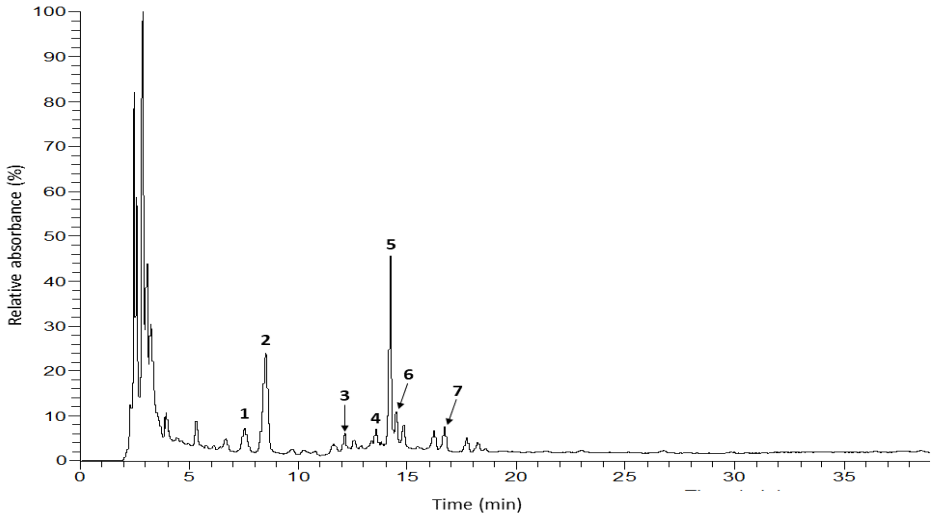

Figure A15. Chromatographic profile of digested BP-A3 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A16. Phenolic compounds and phenylamides profile of the digested BB-A1 sample (gastric phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	10.80	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
3	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
4	12.14	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	13.60	270	477	MS^2: 315 (100), 462 (42), 300 (14); MS^3: 300 (100)	Methyl herbacetin-3-*O*-hexoside
6	16.84	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	N^1, N^5, N^{10}-*tri*-caffeoylspermidine
7	22.37	296, 310	598	MS^2: 462 (100), 452 (42), 478 (41), 342 (14)	N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine (isomer)
8	24.38	295, 310	582	MS^2: 462 (100), 436 (9), 342 (6)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine
9	26.72	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine

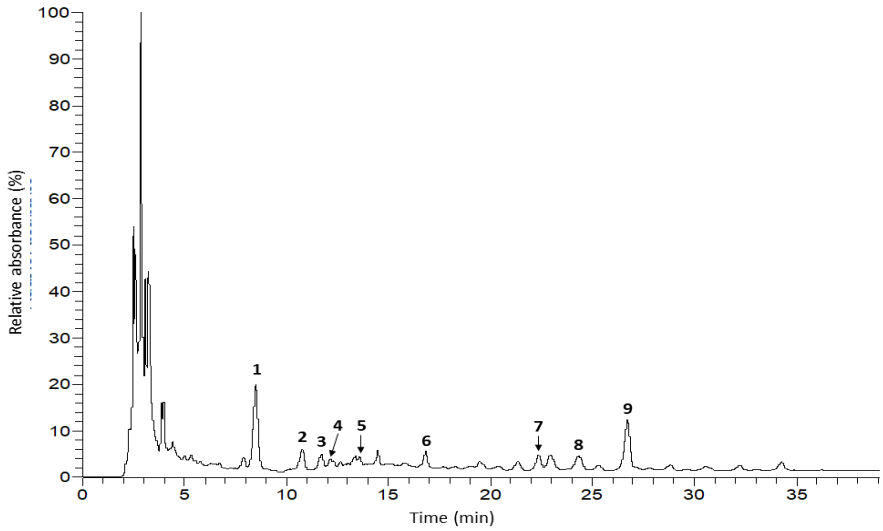

Figure A16. Chromatographic profile of digested BB-A1 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A17. Phenolic compounds and phenylamides profile of the digested BB-A2 sample (gastric phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.89	201, 252, 341	830	–	ND
2	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	10.77	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
4	12.16	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside

5	12.32	211	623	–	ND
6	15.03	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside
7	16.83	296, 319	630	MS^2: 468 (100), 494 (86), 358 (7); MS^3: 332 (100)	*N^1, N^5, N^{10}-tri*-caffeoylspermidine
8	19.48	295, 311	614	MS^2: 494 (24), 478 (100), 452 (76), 358 (22)	N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine (isomer)
9	26.70	296, 310	582	MS^2: 462 (100), 436 (10), 342 (7)	*N^1, N^5, N^{10}-tri-p*-coumaroylspermidine

ND = not detected.

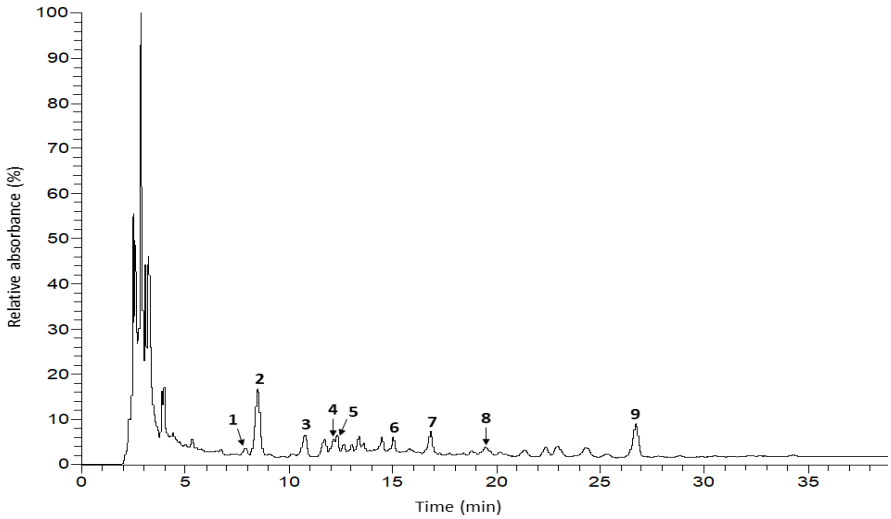

Figure A17. Chromatographic profile of digested BB-A2 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A18. Phenolic compounds profile of the digested BB-A3 sample (gastric phase).

Peak	**t_R (min)**	**λ_{max} (nm)**	**$[M-H]^-$ *m/z***	**MS^n (% base peak)**	**Proposed compound**
1	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	11.73	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	12.17	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
4	12.62	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside
5	15.03	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside

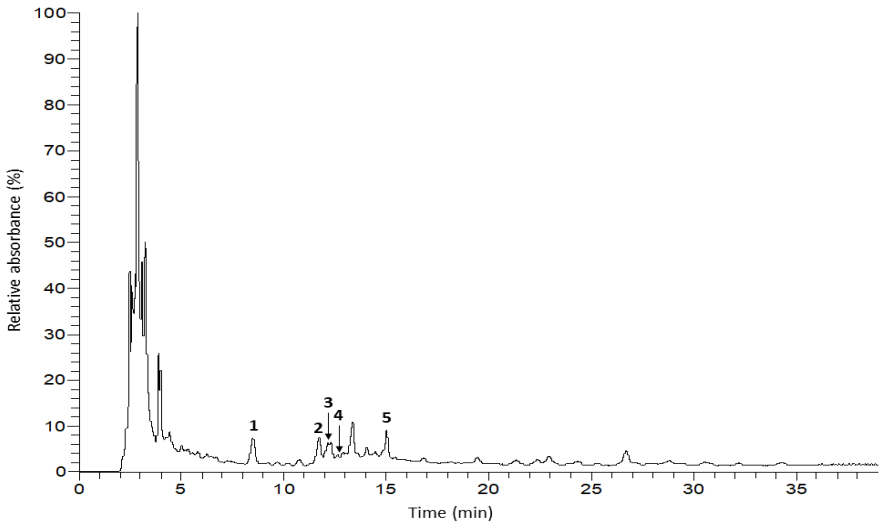

Figure A18. Chromatographic profile of digested BB-A3 sample (gastric phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A19. Phenolic compounds profile of the digested BP-A1 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.91	252, 267, 346	831	MS^2:785	ND
2	11.74	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside[c]
3	12.15	265, 348	593	MS^2: 284 (100), 285 (73), 429 (93)	Kaempferol-*O*-rutinoside
4	12.56	256, 354	463	MS^2: 301 (100)	Quercetin-3-*O*-glucoside
5	13.63	253, 356	669	MS^2: 379 (100), 623 (6), 285 (14)	ND

ND = not detected.

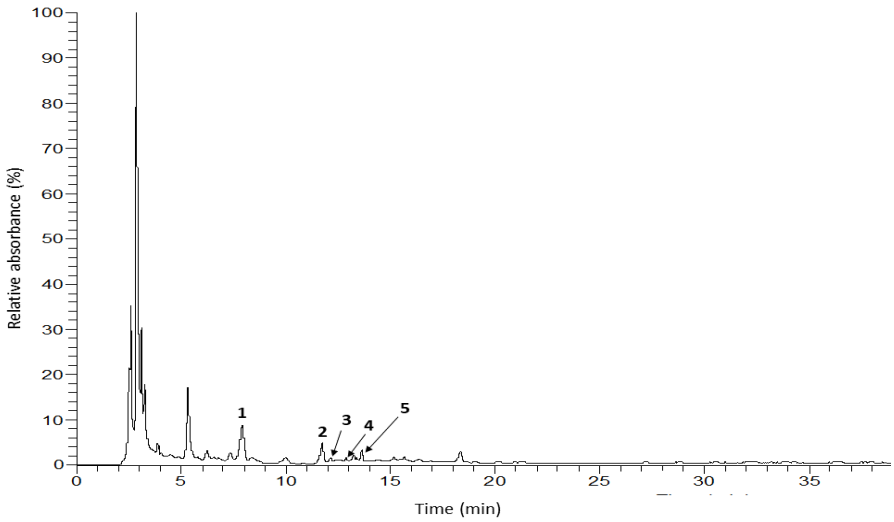

Figure A19. Chromatographic profile of digested BP-A1 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A20. Phenolic compounds profile of the digested BP-A2 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.90	201, 252, 341	830	–	ND
2	11.69	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	13.64	218	668	MS^2: 379, 380, 285	ND

ND = not detected.

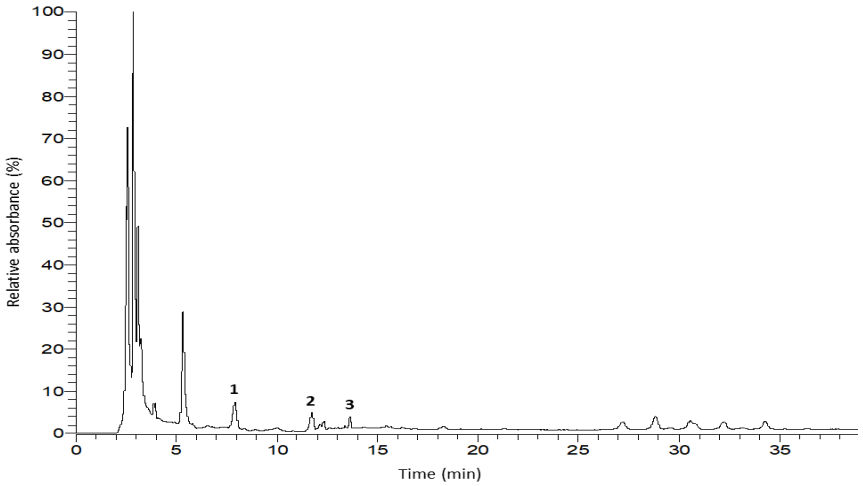

Figure A20. Chromatographic profile of digested BP-A2 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A21. Phenolic compounds profile of the digested BP-A3 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.51	257, 353	625	MS^2: 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin-diglucoside
2	8.50	272, 326sh, 353sh	639	MS^2: 271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	12.14	267, 347	593	MS^2: 284 (100), 285 (69)	Kaempferol-3-*O*-rutinoside
4	14.18	254, 347	447	MS^2: 301 (100)	Quercetin-3-*O*-rhamnoside
5	16.71	255, 354	461	MS^2: 314 (100), 315 (77), 299 (39)	Isorhamnetin-*O*-deoxyhexoside

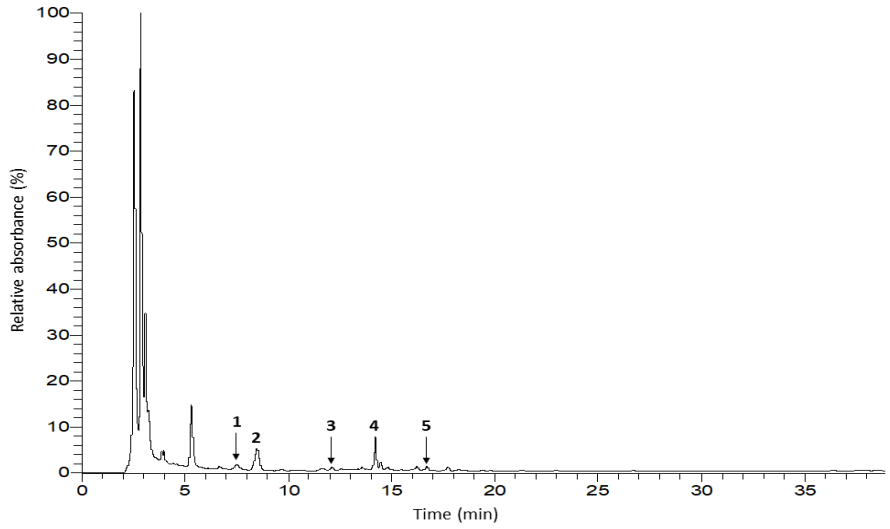

Figure A21. Chromatographic profile of digested BP-A3 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A22. Phenolic compounds profile of the digested BB-A1 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.90	253 st, 348	174	MS^2: 130, 146	ND
2	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	10.79	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
4	11.74	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
5	13.63	270	477	MS^2: 315 (100), 462 (42), 300 (14); MS^3: 300 (100)	Methyl herbacetin-3-*O*-hexoside

ND = not detected.

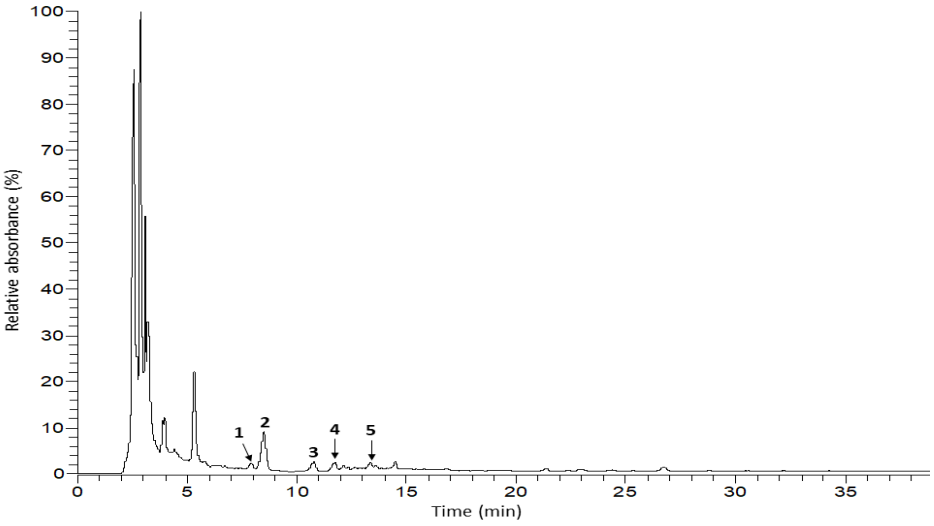

Figure A22. Chromatographic profile of digested BB-A1 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A23. Phenolic compounds profile of the digested BB-A2 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	7.89	201, 252, 341	830	–	ND
2	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
3	10.78	272, 326sh, 353sh	623	MS^2: 271 (10), 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin-*O*-rutinoside
4	12.16	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
5	12.33	211	623	–	ND
6	15.03	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside

ND = not detected.

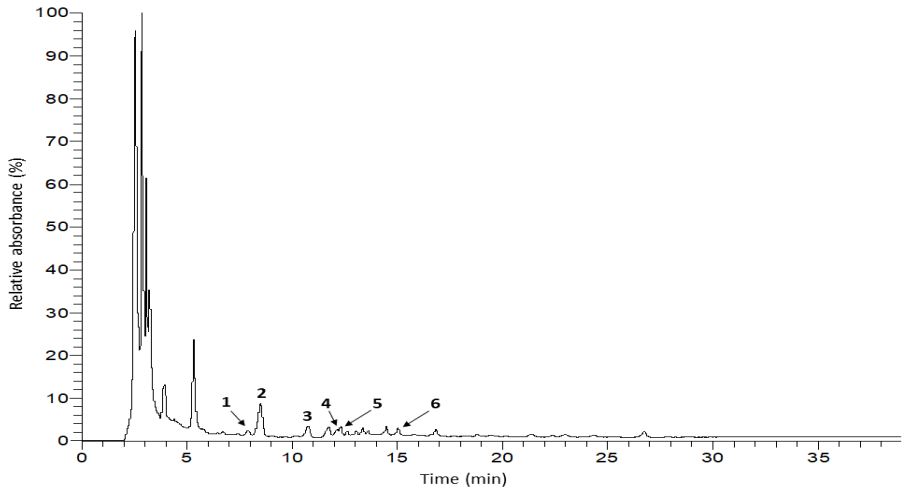

Figure A23. Chromatographic profile of digested BB-A2 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

Table A24. Phenolic compounds profile of the digested BB-A3 sample (intestinal phase).

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Proposed compound
1	8.50	272, 326sh, 353sh	639	MS^2:271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin-*O*-dihexoside
2	11.74	255, 353	609	MS^2: 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin-*O*-pentosyl-hexoside
3	12.16	266, 349	593	MS^2: 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3-*O*-rutinoside
4	15.03	265, 345	533	MS^2: 489 (100); MS^3: 285 (100)	Kaempferol-*O*-malonyl hexoside

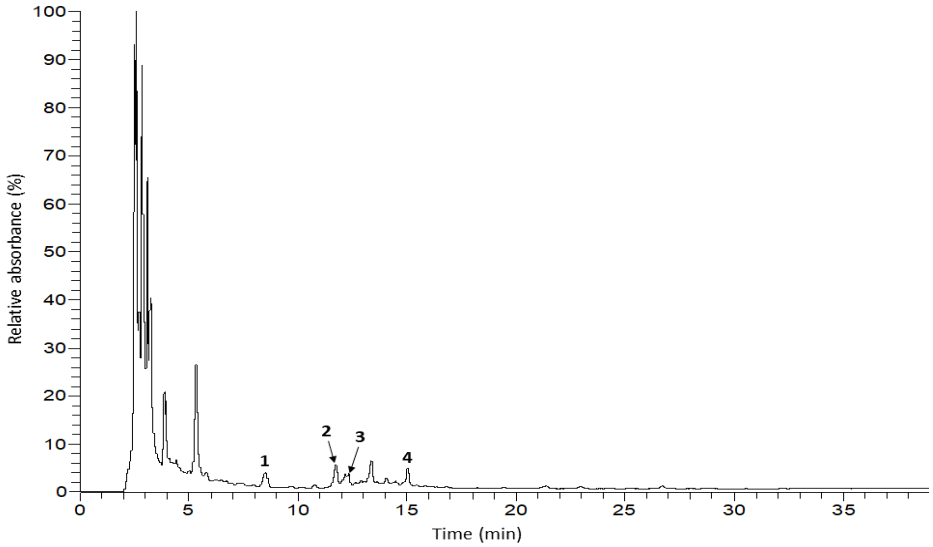

Figure A24. Chromatographic profile of digested BB-A3 sample (intestinal phase) obtained at 280 nm by LC/DAD/ESI-MS^n.

2. List of publications

This thesis work resulted in the following studies:

1- Under review

From the hive to the table: Nutrition value, digestibility and bioavailability of the dietary phytochemicals present in the bee pollen and bee bread

Volkan Aylanc, Soraia I. Falcão, Seymanur Ertosun, Miguel Vilas-Boas*

Abstract

Background

The consumption of natural products has been increasing significantly due to the idea that whether improving nutrition, improves health, general well-being and reduces the risk of developing certain diseases. Bee products, in special bee pollen and bee bread, have demonstrated several nutritional and bioactive properties, which make them functional foods par excellence. Thus, understanding the digestibility and the changes of phytochemicals along the digestive tract, which give bee pollen and bee bread the functional food attribute, is crucial.

Scope and approach

This review describes the digestibility, bioavailability, and absorption behaviors of dietary phytochemicals present in bee pollen and bee bread. It also addresses possible factors that may adversely affect the human health due to its intake and highlights food practices for the industry.

Key findings and conclusions

Many studies have been conducted on bee bread and bee pollen, and these studies have mostly evaluated the nutritional values and the bioactive compounds content of the raw form of these bee products. However, few studies have addressed the nutritional and phytochemical content of bee pollen and bee bread after digestion. Topics such as changes in the digestive tract, post-digestive bioaccessibility, tissue absorption scores and the degree of presence in the circulatory system of the phytochemicals that provide strong biological properties to bee pollen and bee bread, should be taken into consideration in future researches.

Keywords: Bee pollen; Bee bread; Dietary phytochemicals; Digestibility; Bioavailability; Safety status

2- Under submission

Assessment of proteins, sugars and dietary phytochemicals following simulated gastrointestinal digestion of bee pollen and bee bread: Digestibility and bioaccessibility

Volkan Aylanc, Seymanur Ertosun, Andreia Tomás, Paulo Russo-Almeida, Soraia I. Falcão, Miguel Vilas-Boas*

Abstract

Bee pollen and bee bread have always been an excellent natural resource for application in different fields due to their rich nutrient content, containing different bioactive compounds, and health-improving properties. There are extensive studies reporting that both bee products are good sources for a healthy diet, although the data concerning their metabolization on the gastrointestinal tract is quite limited. Herein, the protein content, sugars, and bioactive compounds in both bee products and their bioaccessibility degree at each digestion phase were investigated. The results showed that the protein content in bee pollen and bee bread increased throughout digestion, reaching digestibility rates of 69% and 75%, respectively, whereas a decrease in individual sugars occurred at different rates. The bioactive compounds decreased significantly after digestion and had 18% and 25% of bioaccessibility of each. This study demonstrated that the macronutrients and bioactive compounds in both bee products may be influenced in different ways through the gastrointestinal tract.

Keywords: Bee pollen; bee bread; in-vitro digestive; digestibility; bioaccessibility

3- In preparation stage

Bee pollen and bee bread as nutritional and functional food sources: evaluation of macro and micronutrients and bioactive compounds content

Volkan Aylanc, Soraia I. Falcão, Miguel Vilas-Boas*