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# Differentiation of bee pollen origin through phenolic and volatile profiles

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## ABSTRACT

Nowadays, bee products are notably commended by consumers for medicinal and dietary purposes. Bee pollen is particularly used as a precious source of nutritious compounds like proteins (which includes enzymes), carbohydrates, lipids, minerals as well as a large range of secondary plant metabolites (polyphenols, carotenoids, tocopherol and vitamins). According to the literature, bee pollen possesses great potentialities such as antioxidant, anti-inflammatory, antibacterial activities, among other . Therefore, it can be used as a food supplement to promote the human body resistance against numerous diseases including cancer. However, the bee pollen composition varies depending on the botanical and the geographical origin, which represents the major difficulty in the application of this matrix.

The aim of the present study was to differentiate bee pollen origin through the phenolic and volatile profiles of samples from Portugal and Morocco. Also, the contribution of their phenolic compounds to the antioxidant and cytotoxic activities were investigated. After the determination of the total phenolics and flavonoid content analysis through UV-Vis analysis, the phenolic composition was assessed by liquid chromatography coupled to mass spectrometry (LC-MS). The volatile profile of the samples was access through gas chromatography coupled to mass spectrometry (GC-MS) after solid-phase microextraction (SPME). The antioxidant properties were measured by DPPH and ABTS scavenging activity and reducing power assays. Finally, the cytotoxicity of the extracts was examined employing different human tumor cell lines.

Our results showed that bee pollen has a rich phenolic profile. Samples from Portugal displayed diverse phenylamides and flavonoid glycosides, while the Moroccan ones presented mostly flavonoid glycosides, phenylamides and some phenolic acids, except M7 which contained important phenylamide quantity. Concerning volatile compounds, plenty of compounds were detected majorly aldehydes, alcohols and some ketones. Some samples presented a high antioxidant capacity particularly the chestnut bee pollen from Bragança. In terms of anti-carcinogenic activity, only P1, Asteraceae *carduus* bee pollen, exhibited a remarkable anti-tumor potential against MCF-7 (breast carcinoma). Overall, Bee pollen samples belonging to the same floral origin presented similarities in both phenolic and volatile compounds profiles. Also, some detected phenolic and volatile compounds can be considered as fliral and geographical biomarkers.

**Keywords:** Bee pollen, Phenolic compounds, Volatile compounds, Antioxidant activity, Cytotoxicity Activity.

**RESUMO**

Atualmente, os produtos apícolas são especialmente apreciados pelos consumidores para fins medicinais e dietéticos. O pólen de abelha é particularmente usado como uma fonte valiosa de compostos nutritivos como proteínas (que incluem enzimas), hidratos de carbono, lipídos, minerais, além de uma grande variedade de metabolitos secundários de plantas (polifenóis, carotenóides, tocoferóis e vitaminas). De acordo com a literatura, o pólen de abelha possui grandes potencialidades, tal como atividades antioxidante, anti-inflamatória e antibacteriana. Portanto, ele pode ser usado como um complemento alimentar para promover a resistência do corpo humano contra inúmeras doenças, na qual se inclui o cancro. No entanto, a composição do pólen de abelha pode variar de acordo com a origem botânica e geográfica, representando esta a maior dificuldade na aplicação desta matriz.

O objetivo do presente estudo foi diferenciar a origem do pólen de abelha através dos perfis fenólico e volátil de amostras de Portugal e Marrocos. Também foi investigada a contribuição destes compostos fenólicos para as atividades antioxidante e citotóxica. Após a determinação do conteúdo em fenóis e flavonóides total, feita por análise UV-Vis, a composição fenólica foi avaliada por cromatografia líquida acoplada à espectrometria de massa (LC-MS). O perfil volátil das amostras foi determinado por cromatografia gasosa acoplada à espectrometria de massa (GC-MS) após micro-extração em fase sólida (SPME). As propriedades antioxidantes foram avaliadas pelos ensaios do DPPH e do poder redutor. Finalmente, a citotoxicidade dos extratos foi estudada usando diferentes linhas celulares tumorais humanas assim como células não tumorais.

Os resultados mostraram que o pólen de abelha possui um perfil rico em compostos fenólicos. As amostras de Portugal apresentaram diversas fenilamidas e flavonóides glicosilados, enquanto as com origem em Marrocos apresentaram maioritariamente flavonóides glicosilados, fenilamidas e alguns ácidos fenólicos, com exceção para a M7 que continha uma importante quantidade de fenilamidas. Em relação aos compostos voláteis, uma grande variedade de compostos foi detectada, como aldeídos, álcoois e cetonas. Algumas amostras apresentaram uma elevada capacidade antioxidante em particular o pólen de abelha de castanheiro. Em termos de atividade anticancerígena, apenas a P1, pólen de abelha de Asteraceae *Carduus*, exibiu um potencial antitumoral notável contra MCF-7 (carcinoma de mama). De maneira geral, as amostras de pólen de abelha pertencentes à mesma origem floral apresentaram

semelhanças nos perfis de compostos fenólicos e voláteis. Além disso, alguns compostos fenólicos e voláteis detectados podem ser considerados como biomarcadores flirais e geográficos.

**Palavras-chave:** pólen de abelha, compostos fenólicos, compostos voláteis, atividade antioxidante, citotoxicidade.

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## List of abbreviations

AGS: **S**tomach **G**astric **A**denocarcinoma

BP: **B**ee **P**ollen

CaCo2: Epithelial **Co**lorectal adeno**ca**rcinoma

DPPH: 2,2-**di**p**h**enyl-1-**p**icryl**h**ydrazyl

DAD: **D**iode-**A**rray **D**etection

ESI: **E**lectro**s**pray **I**onization

FRAP: **F**erric **R**educing **A**ntioxidant **P**ower

GAE: **G**allic **A**cid **E**quivalent

GC: **G**as **C**hromatography

GI: **G**rowth **I**nhibition

HeLa: Cervical carcinoma

hFOB: **h**uman **F**etal **O**steo**b**lastic

HPLC: **H**igh-**p**erformance **L**iquid **C**hromatography

ICP-OES: **I**nductively **C**oupled **P**lasma-**O**ptical **E**mission **S**pectrometry

LC: **L**iquid **C**hromatography

MCF-7: Breast adenocarcinoma

MS: **M**ass **S**pectrometry

MR: **M**o**r**occo

NCI-H460: **n**on-small **c**ell **l**ung cancer

QE: **Q**uercetin **E**quivalent

PC: **P**henolic **C**ompounds

PDMS/DVB: **P**oly**di****m**ethyl**s**iloxane/**Di**vinyl**b**enzene

PT: **P**or**t**ugal

R%: **R**elative Percentage

RPLC: **R**eversed-**P**hase **L**iquid **C**hromatography

RPM: **R**otation **p**er **m**inute

RT: **R**etention **t**ime

SPME: **S**olid **P**hase **M**icro**e**xtraction

TEAC: **T**rolox **E**quivalent **A**ntioxidant **C**apacity

TFC: **T**otal **F**lavonoid **C**ontent

TPC: **T**otal **P**henolic **C**ontent

UPLC: **U**ltra high-**p**erformance **L**iquid **C**hromatography

VOC: **V**olatile **O**rganic **C**ompound

w: **w**eight

# 1. Introduction

In the course of evolution, a special relationship has been established between plants and bees. Bee pollen is composed of hundreds and even thousands of plant pollen that have been harvested by bees. The insects use their saliva secretions and plant nectar to agglomerate the grains and then transport it to their hives (De-Melo and de Almeida-Muradian, 2017).

It is widely known that bee pollen presents a source of nutrients particularly proteins, amino acids, carbohydrates, vitamins, polyphenols, among other… (Ketkar *et al.*, 2015). Consequently, it gained attention and recently its consumption has increased (De-Melo and de Almeida-Muradian, 2017). Also, it could be successfully used as a potential therapeutic or synergetic agent in the treatment of several cases of cancer, which presents the globally second leading cause of mortality (Fatrcová-Šramková et al., 2013; Mărgăoan et al., 2019; Who. int, 2019). The cancer drug usage is in progress, despite their adverse reactions or recurrence. Hence, to avoid such side effects, chemotherapy is currently associated with "natural products" which contribute to the reduction or even the suppression of adverse impacts (Mărgăoan et al., 2019). Also, free radical-scavenging and metal-chelating activities of bee pollen were studied, proving that it decreases the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation (Fatrcová-Šramková *et al.*, 2013).

However, the composition and the biological potential of bee pollen are variable depending on its geographic and botanical origins, soil type, seasonal and environmental factors as well as beekeeping activities (Ulusoy and Kolayli, 2014).

Thus, one of the elementary aspects of bee pollen's commercial quality is its botanical origin declaration. Accordingly, in this study, the floral origins of pollen samples from Morocco and Portugal were identified, as well as the phenolic and volatile compounds profile of each sample was investigated. Besides that, the contribution of the bioactive compounds to the antioxidant and anti-tumor activities of bee pollen was studied.

In this context, the present work has as main objectives:

- Identification of the botanical origin (palynological analysis) of 8 bee pollen samples from Morocco and 7 samples from Portugal through palynological analysis.
- Determination of the total phenolics and flavonoids content
- Evaluation of the antioxidant activity of the phenolic extracts
- Characterization of the samples in terms of phenolic compounds through LC/DAD/ESI-$MS^n$;
- Optimization of the SPME volatiles extraction from the bee pollen samples and profiling through GC-MS.
- Screening in vitro of the bee pollen samples against several human tumor cell lines.

# 1. Literature review

## 1.1. Apiculture and bee products

Apiculture or beekeeping is "the science studying bees' life, keeping and care in order to obtain and use the apicultural products and to pollinate agricultural crops" (Grigoras, 2018). It supplies bee products which have a high nutritive, energetic value, and curing effect with an important role in human health. Beekeeping improves agriculture by assuring pollination of the entomophilous plants, as a result, it rises crops, fruits, vegetables, and grains harvests (Grigoras, 2018). Since apiculture adds activity diversification in the countryside, it increases the local natural and human resources, offering an alternative income for the indigenous population in developing regions with almost little investment (Grigoras, 2018)

Honey and wax are the most known primary bee products, however, pollen, propolis, royal jelly, bee venom, bees, and their larvae are also commercial primary products of beekeeping (Fao.org, 2019). Bee products are also used as ingredients or supplements in the cosmetic and agri-food industry which consequently will increase the product's commercial value (Fao.org, 2019). The predominant bee products that are consumed by humans are described below.

### 1.1.1. Honey

According to Codex Alimentarius (2001), "honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature". From a chemical point of view, honey is considered as a natural mixture of sugars and water together with other minor constituents such as minerals, vitamins, amino acids, organic acids, flavonoids, and other

phenolic compounds and aromatic substances (Alvarez-Suarez, 2017a).The honey composition and especially its bioactive components are determined by the botanical and geographical origin, and also it can be dependent on the storage conditions and time (Alvarez-Suarez, 2017a).

Figure 1: Different colored kinds of honey of unifloral and multifloral origin (Krell, 1996).

### 2.1.2. Propolis

Propolis is a sticky complex mixture typically composed of 50% plant resin, 30% waxes, 10% essential and aromatic oils, 5% pollen, and 5% other organic substances (Huang et al.,2014). Usually propolis color varies from green to brown and reddish which is determined by its botanical origin. Also acknowledged as “bee glue”,(Maria Graça Miguel & Figueiredo, 2017) it is considered as a building and sheltering material which bees apply to maintain their hive: sealing holes, covering surfaces and closing gaps, and keeping moisture as well as temperature stable (Siheri et al., 2017).

Figure 2 : Raw propolis (Yucel et al., 2017)

Propolis is then affirmatively known for being a robust chemical barrier against bacteria, viruses, and other pathogenic microorganisms that can penetrate to the colony providing a sterile environment (Siheri *et al.*, 2017; Wagh, 2013). In addition, bees utilize propolis for embalming substances, mummifying attackers who have been killed but heavy to remove from the colony (Siheri et al., 2017).

### 2.1.3. Royal jelly

Royal jelly is a secretion full of proteins and lipids which is made by the nurse bees' hypopharyngeal and mandibular glands (Dimou *et al*., 2007; Pasupuleti et al., 2017). It is a sticky and creamy product, moderately soluble in water, approximately acidic (pH 3.1–3.9), with a density of 1.1 g/mL, a color ranging from white to yellow, and has a slightly pungent odor and taste. Royal jelly is principally composed of water (60–70%), proteins (9–18%), carbohydrates(7–18%), lipids (3–8%), and other minority components like minerals (0.8–3%), vitamins, phenols and amino acids (Maria G. Miguel & El-Guendouz, 2017).

Nurses feed young larvae of workers and drones for the first 3 days with the royal jelly yet the queen bee consumes it throughout its whole life. This superfood is the major cause for the queen bee's longevity compared to the other bees (Pasupuleti et al., 2017).

Furthermore, both traditional and modern medicine make the most of royal jelly due to its biological benefits in particular antibacterial, antitumor, antiallergy, anti-inflammatory, and immunomodulatory effects. Royal jelly has a considerable commercial value, being used in a lot of sectors like the pharmaceutical, food industries, and even in the cosmetic field (Dimou *et al*., 2007; Pasupuleti et al., 2017).

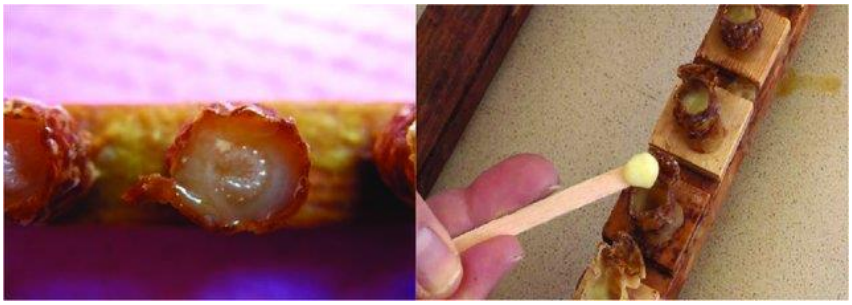

Figure 3: Royal jelly in cells (Yucel, Topal, and Kosoglu, 2017)

### 2.1.4. Bee venom

Female worker bees produce bee venom and use it as a defense tool against predators to protect themselves or their colony. It's a transparent odorless liquid which contains a protein hydrolytic mixture with a pH ranging from 4.5 to 5.5. One drop of bee venom mainly consists of 88% water and only 12% of dry venom. The latter weighs around 0.1 micrograms, composed of a great complex blend of peptides such as melittin, adolapin, and apamin. Enzymes are also present in this mixture, most importantly PLA2 and other compounds like bioactive amines and minerals (Wehbe et al., 2019).

The use of this natural toxin, in specific areas of the human body, is called bee venom therapy which is widely used in complementary and alternative medicine (Pak, 2017). Several studies estimate the curative efficiency of the toxin in treating not only human inflammatory diseases but central nervous system diseases too, suchlike Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, as well as other numerous conditions (Wehbe et al., 2019).

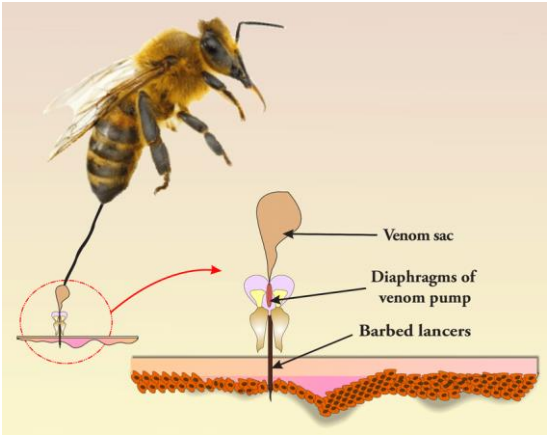


Figure 4 : Bee venom delivery (Elieh Ali Komi et al., 2018)

### 2.2. Bee pollen

Bee pollen is a product generated by bees to serve as a nutrient source for the colony's growth and maintenance. The insects gather pollen grains which are the male reproductive units (gametophytes) of flowering plants. While they fly, bees amplify on their body a positive static-electric charge. Since the pollen dust is negatively charged, it will be attracted to the insect's body and stick to it (Beeculture.com, 2019).

Thereafter, bees agglutinate it using its salivary secretions, nectar, and/or honey to make up pollen loads which generally varies between 1.4 and 4 mm in size. (De-Melo & Almeida-Muradian, 2017).

Afterward, bees carry the loads in pollen baskets on their back legs to the hive. Beekeepers harvest fresh pollen pellets employing pollen traps, which are devices made of a grid and fixed at the entrance of the hive (Bogdanov and Science, 2016).

Figure 5: Bee harvesting pollen dust ("Sweet success: how bees choose which pollen to collect", 2016)

High humidity (20-30% of fresh pollen weight) makes the bee pollen an ideal matrix for microbial contamination and spoilage, consequently, it requires a conservation process: drying and adequate storage. (Bogdanov & Science, 2016; Rzepecka-Stojko et al., 2015). From that point, bee pollen becomes the raw material for nutritional, pharmaceutical as well as cosmetic use (Rzepecka-Stojko et al., 2015).

(a)

(b)

Figure 6 : (a) Pollen trap (Greenwood, 2019) (b) Pollen pellets (Yucel, Topal, and Kosoglu, 2017)

The bee-collected pollen, which is made predominantly or entirely from a single botanical source, is called monofloral or unifloral bee pollen. However, if bee pollen lacks the predominance of one plant species it is classified as multiflora bee pollen (De França Alves & de Assis Ribeiro dos Santos, 2016). When harvesting the pollen, beekeepers obtain a mixture of several botanical origins pollen. A color-based separation is needed to get the monofloral bee pollen since it has a constant composition which makes it effectively used in medicine and nutrition (Bogdanov, 2016).

### 2.2.1. Composition

From the moment in which the honeybees introduce their secretions to plant pollen, this latter gain certain unique characteristics which make it special from the pollen dispersed by the wind or collected by hand (Ares et al., 2018).

Bee pollen contains several nutrients and bioactive compounds, mostly proteins, which include enzymes and both essential and non-essential amino acids. As a matter of fact, bee pollen is indicated as the "only perfectly complete food", since it provides all the essential amino acids for the human organism. Numerous carbohydrates are also found in this bee product, including monosaccharides, polysaccharides, soluble and non-soluble fibers. Moreover, bee pollen contains lipids, such as fatty acids, sterols, and triglycerides. Also, several minority compounds, for example, minerals and vitamins (water and fat-soluble), and other various bioactive compounds, like the phenolic and volatile compounds can be found in this bee product (Ares et al., 2018).

Lately, the interest in the extraction and the determination of the effective compounds of this matrix has increased due to the high number of published research papers that deal with this issue. A lot of bioactive compounds have been examined in bee pollen, mainly proteins, peptides and amino acids, lipids, carbohydrates, and phenolic compounds (Ares et al., 2018; Feas et al., 2012).

The chemical composition of bee pollen fluctuates considerably, depending on the pollen grains' botanical and geographical origin. Thus, the composition of the grains undergoes the influence of the climate conditions, the soil, plant nutritional state, and even its age. Significant variations in pollen composition were found in the same plant species according to the region, year's season, and even between years (De-Melo & de Almeida-Muradian, 2017).

#### 2.2.1.1. Carbohydrates

Carbohydrates are the major fraction of bee pollen, ranging from 15% to 60% of the matrix dry weight (Ares et al., 2018). Bee pollen carbohydrates are influenced, like the other components, by the botanical origin of the plant pollen, in addition, they are also considered as floral discrimination markers. Biologically, they play several crucial roles, polysaccharides save energy and are structural substances, while monosaccharides provide energy for the metabolism. The main characteristic that makes bee pollen different from plant pollen, is the reducing sugars predominance over the non-reducing

ones (Ares et al., 2018). In bee collected pollen, fructose is the most abundant sugar succeeded by glucose, whereas other carbohydrates can also be found such as sucrose and dietary fibers (Ares et al., 2018).

Normally, carbohydrates are analyzed by gas chromatography and high-performance liquid chromatography, but due to their elevated molecular weight and the considerably hydrophilic nature, sometimes they are difficult to be analyzed. As a result, it's important to develop effective, inventive, and proper detection methods for oligosaccharides and polysaccharides in this beehive product (Li et al., 2018).

#### 2.2.1.2. Proteins, peptides, and amino acids

Proteins are the second most abundant component in dry bee pollen, making up around 14–30% (W/W). Besides that, a total of 20 essential amino acids were identified in bee pollen. Usually, this bee product is plentiful of proline, glutamic acid, and aspartic acid, depending on that abundance on the different geographic and botanical origins of the samples (Li et al., 2018). Additionally, bee pollen collected from spring flowers tends to be richer in protein content than summer or autumn flowers (Ares et al., 2018).

Amino acid's presence in bee pollen overcomes the question of nutrition since it can serve as a freshness marker and an indication of the adequacy of the drying and storage process. Also, a specific amino acid profile could be correlated with specific plant species and be used to determine the botanical and the geographical origin of samples (Ares et al., 2018; Li et al., 2018). Consequently, bee pollen is considered an extraordinary alternative dietary supplement, especially for vegetarians, as a food enhancer thanks to its high levels of proteins and amino acids (Fao.org, 2019).

Total protein content has been determined in many publications using the nitrogen measurement by a Kjeldahl method (Ares et al., 2018). In contrast, techniques such as HPLC, LC-MS, and ion-exchange chromatography are applied for amino acid analysis (Li et al., 2018).

#### 2.2.1.3. Lipids

Pollen grains need lipids as an essential nutrient for development, energy, reserve, and performance. It can present between 1% and 20% of bee pollen dry weight. This bee product can be a potential source of lipids to humans since they are not able to biosynthesis all the essential fatty acids which must be obtained from the diet (Ares et al., 2018).

Among lipids in bee pollen, we can mention essential fatty acids that are (EFAs), present in an amount approximately of 4%, which includes linoleic, $\gamma$-linoleic, and arachidonic acid. Also, phospholipids, phytosterols, and carotenoids were detected in the amount of 1.5% and 1.1% respectively (Komosinska-vassev et al., 2015; Li et al., 2018).

Lipids in bee pollen can be profiled by ultra-performance liquid chromatography coupled to tandem with mass spectrometry however due to its complex structure and the existence of several isomers. make it complicated to recognize certain classes of lipids such as sphingolipids with this chromatography method (Ares et al., 2018; Li et al., 2018). Gas chromatography with flame ionization (GC-FID,) with a previous trans-esterification of the sample, is a very used technique for fatty acids determination in bee pollen (Feas et al., 2012).

#### 2.2.1.4. Vitamins

Vitamins are a diversified compounds group that possesses several functions as cell regulators in tissue differentiation and growth as well as mineral metabolism. In addition, numerous vitamins act as enzyme cofactor precursors or can present antioxidant activity. As claimed in the existing literature, bee pollen has plenty of vitamins, above all group-B vitamins, being the most abundant vitamin B3 and its two forms: nicotinamide and niacin. Also, bee pollen contains vitamins C, D, E, and some carotenoids suchlike carotene which may be pro-vitamin A (Ares et al., 2018).

As verified for other bioactive molecules, vitamin concentration in bee pollen varies according to the plant source. Therefore, it can provide an indication of the beehive product's botanical origin (Li et al., 2018). However, thermally unstable compounds such as vitamins A and C could be degraded during the thermal processing of bee pollen (Ares et al., 2018).

Several analytical approaches can be used to detect these compounds in bee pollen, depending on the vitamin's properties. For instance, to identify the group-B vitamins, the reversed-phase liquid chromatography coupled with a fluorescence detector (RPLC-FLD) was used as the most effective analytical method. Yet, to analyzed the presence of tocopherols, chemical compounds class that have vitamin E activity, normal-phase liquid chromatography with a silica column was mentioned by the literature (De-Melo & Almeida-Muradian, 2017; Santos-Buelga & González-Paramás, 2017).

#### 2.2.1.5. Minerals

Bee pollen is a valuable source of indispensable minerals suchlike potassium, calcium, magnesium, sodium, sulfur, iron, copper, manganese, phosphorus, zinc, chromium, nickel as well as selenium. Minerals are fundamental for physiological processes and for good metabolic pathways regulation. Therefore, minerals must be sufficiently consumed to ensure homeostasis, functionality, and cell production, otherwise, their deficiency may provoke illness (Ares et al., 2018).

As mentioned by the literature, bee pollen is considered an important food for balanced diets in terms of mineral content, since it provides the zinc, copper, iron, and potassium/sodium ratio needed by the human organism (Ares et al., 2018). Despite the beneficial effects of the mineral content in bee collected pollen, other detrimental minerals known as contaminants are present too, among them barium, mercury, cadmium, and lithium. Those heavy metals can compete with the other essential trace elements, such as copper along with iron, and bind with biological systems. Pesticides, fertilizers, and other chemicals used in the farming sector are the main sources of heavy metals (Ares et al., 2018).

Like the other bee pollen components, mineral content varies depending on the geographical region, soil type, botanical origin together with the year season of the collection. Yet, minerals are not influenced by the processing and storage conditions like the other macronutrients of bee pollen (De-Melo & Almeida-Muradian, 2017).

To detect minerals in this matrix, several reliable methods can be used as inductively coupled plasma-optical emission spectrometry (ICP-OES), total reflection X-ray fluorescence (TXRF), and atomic absorption spectrometry (ASS) (Li et al., 2018).

#### 2.2.1.6. Phenolic compounds

Phenolic compounds are a large class of phytochemicals that are abundant micronutrients in the human diet and play a major role in plant protection against biotic and abiotic stresses (Rajbhar, Dawda, and Mukundan, 2015; Kostić *et al.*, 2019). They stand for a wide range of similarly structured compounds, around 8000 molecules, that have at least one aromatic ring with at a minimum one hydroxyl group (Garcia-Salas et al., 2010). Several researchers subdivide those molecules into three major subclasses: flavonoids, phenolic acids, and stilbenoids. The vast majority of them are naturally

associated with mono- or polysaccharides or with functional derivatives such as esters or methyl esters (Garcia-Salas et al., 2010).

The most extensive phenolics in the human diet are flavonoids as well as phenolic acids (cinnamic and benzoic acids). Flavonoids may be detected in plants in the form of aglycones, Figure 7 (a), or glycosides, figure 7 (b), which provide the color of leaves, fruits, and flowers (orange, carmine, blue). These biomolecules occur not only in vegetables and fruits but also can be found in nuts, cereals, spices, medicinal plants, and beverages (Garcia-Salas et al., 2010; Rzepecka-Stojko et al., 2015).

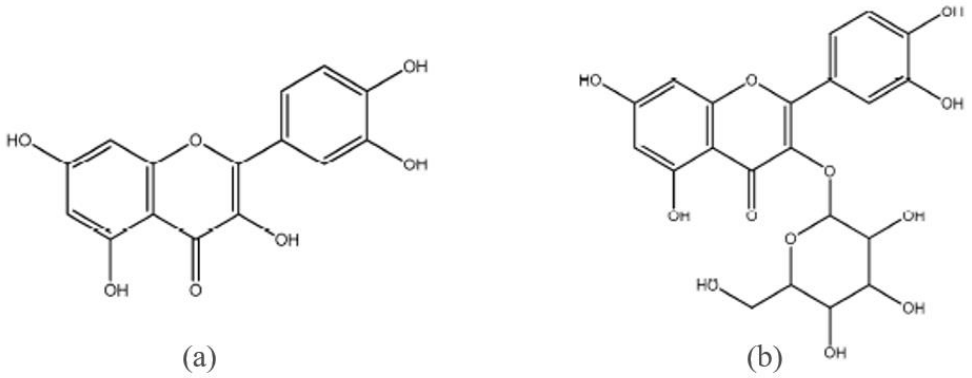


Figure 7: (a) Quercetin aglycone form (b) Quercetin glucoside (Sobral et al., 2017)

The biological activity of phenolic compounds appears to be related on one hand to the intrinsic capacity of free-radical scavenging as hydrogen or electron-supplying agents and on the other hand to the ability to complex metal ions which catalyze reactions of oxidation (Rzepecka-Stojko et al., 2015; Şabanoğlu et al., 2019). Thanks to the ring structure, phenolic compounds, especially flavonoids, show lipophilic properties that protect the lipid pollen membrane. Also, they are involved in interactions with receptors, in the modulation of the activity of certain enzymes, in the mode of signal transduction, and regulation of the cellular cycles (De-Melo & Almeida-Muradian, 2017; Rzepecka-Stojko et al., 2015). Bee pollen is then considered as a source of antioxidants that may be exploited to improve the properties of pharmaceuticals, cosmetics, and even foods, owing to the presence of double bonds as well as the location of hydroxyl groups on the aromatic ring (Rzepecka-Stojko *et al.*, 2015; Şabanoğlu, Gökbulut, and Altun, 2019).

Researchers hypothesized that every single plant species has a specific phenolic profile. In this way, based on that, it's easier to identify the botanical species from which bee pollen was collected as well as its biological activity. Certainly, agricultural and

genotypic dissimilarities along with climatic conditions (temperature, light intensity, and hydrological stress) and soil type, must be considered while associating the botanical origin, the biological activity, and the phenolic profile (De-Melo & Almeida-Muradian, 2017).

Several studies demonstrated that bee pollen is mainly rich in phenolic acid derivatives, glycosylated flavonoids, and aglycones which are the hydrolysis product of alpha and beta-glucosidases (enzymes secreted by bees) (Rocchetti et al., 2019). The phenolic profile of bee pollen is very variable. A study has been undertaken concerning the phenolic profile of monofloral samples from Brazil and showed that Myrcia pollen (99.5%) contained as predominant compounds ellagic acid and myricetin. However, glycosylated anthocyanin was the major component of the Alternanthera pollen (94.2%) sample (De-Melo et al., 2018).

A diverse range of analytical approaches has been employed for phenolic compounds analysis. The classical method commonly associates liquid chromatography separation (HPLC) with UV-Vis detection (DAD) or with mass spectrometer detectors (MS). Recently, ultra-high-performance liquid chromatography (UPLC) has been used to analyze phenolic compounds due to the high speed and high resolution of this technique. As stated by the literature, capillary electrophoresis makes a good alternative separation technique to HPLC, its principal advantage being time-saving and the need for smaller volumes of solvent (Fanali et al., 2013). Considerable attention has been paid to the invention of environmentally friendly and cheap technologies able to provide fast analysis. One of them is the miniaturized version of LC, capillary-/nano-LC, which offers good efficiency, good resolution, and short analysis time with limited use of mobile/stationary phase, therefore, a low environmental impact (Fanali et al., 2013).

#### 2.2.1.7. Volatile compounds

Volatiles are commonly assumed as the compounds emitted from flowers to attract pollinators as well as preserve the plant's reproductive parts from enemies (Dudareva, Pichersky, and Gershenzon, 2004). The literature data about volatiles in bee pollen is very scarce and insufficient. Pollen has an integral part of honey, therefore, volatile compounds detected in honey can be similar to the ones detected in bee pollen (Kaškonienė et al., 2015). Besides, the volatile profile of honey and bee pollen depends on the botanical origin which provides a unique aromatic and organoleptic properties that can alter consumer preferences (Gianelli Barra et al., 2010; Kaškonienė et al., 2015). Identified

volatiles in bee pollen can be classified in various chemical families such as alcohols, acids, aldehydes, esters, ketones, linear and branched terpenes, as well as saturated and unsaturated hydrocarbons (Gianelli Barra et al., 2010; Kaškonienė et al., 2015). Those aromatic compounds have relatively low molecular weight and can be detected with minor concentrations. However, they are spotted as a very complex mixture of volatiles with numerous physicochemical properties, levels of stability as well as biological functionalities (Manyi-Loh, Ndip, and Clarke, 2011; Bianchin *et al.*, 2014). Table 1 shows results from the literature of predominant volatile compounds extracted from honey and utilized as floral markers to identify its botanical origin.

Table 1: Identified floral markers used to determine honey's botanical origin (Manyi-Loh, Ndip, and Clarke, 2011).

| **Honey type** | **Country** | **Floral marker** | **Employed method** |
|---|---|---|---|
| **Eucalyptus** | Spain | 3-caren-2-ol<br>p-cymene and its derivate alcohol | Micro-simultaneous Steam Distillation-solvent Extraction coupled to GC-MS |
| **Citrus** | | Linalool oxide<br>lilac alcohol and lilac aldehyde | |
| **Lavender** | | Nerolidol oxide | |
| **Chestnut** | Italy | Aminoacetophenone | SPME-GC-MS |
| **Lime tree** | | p-methylacetophenone<br>carvacrol<br>8-p-menthen-1,2-diol | |
| **Citrus** | | Methyl anthranilate<br>limonene diol | |
| **Thyme** | Greece | 3-hydroxy-4-phenyl-2-butanone<br>3-hydroxy-1- phenyl-2-butanone | Ultra Sound Extraction coupled to GC-MS |

Multiple techniques have been performed to evaluate and quantify volatile compounds that are responsible for the aroma. In the case of bee pollen, gas chromatography coupled to mass spectrometry (GC-MS) is considered a useful technique for this analysis due to the efficiency and sensitivity of the separation (Bianchin et al., 2014). For better separation and detection, volatiles are adsorbed from the sample in headspace through solid-phase micro-extraction (SPME) (Gianelli Barra et al., 2010).

### 2.2.2. Biological properties

Bee-collected pollen is extremely rich in bioactive substances, hence it offers multidirectional effects suchlike anti-cancerogenic, anti-inflammatory, antibacterial effects. The main biological activities of bee pollen are shown in Table 2.

Table 2 : Bioactive properties of bee pollen (Ares et al., 2018; De-Melo & de Almeida-Muradian, 2017; Komosinska-vassev et al., 2015; Rzepecka-Stojko et al., 2015)

| **Bioactive properties** | **Mechanism of the biological activity** |
|---|---|
| **Antioxidative** | Scavenging hydroxyl radicals, complexing metals by the hydrophilic antioxidants (vitamin C + polyphenolic compounds) and the hydrophobic antioxidants (vitamin E + carotenoids) |
| **Cardioprotective** | Effects on an enzyme converting angiotension1 to angiotensin2 (ACE° by the essential unsaturated fatty acids, vitamin E, phytosterols, phospholipids, and flavonoids). |
| **Hepatoprotective** | Reduction of lipofuscin and detoxifying activity by decreasing the oxidative stress markers. |
| **Antibacterial** | Powerful properties against pathogenic Gram+ and Gram- bacteria and pathogenic fungi by the presence of flavonoids, phenolic acids, polyamides, and some fatty acids. |
| **Anti-inflammatory** | Inhibition enzymes that turn arachidonic acid into prostaglandin and leukotrienes by the action of flavonoids, phenolic acids, fatty acids, and phytosterols. |
| **Anti-carcinogenic** | Cytotoxic properties against tumors through increasing the activity of the caspase-3 enzyme which leads to the prostate cancer cells apoptosis.<br>Decrease the risk of the disease in the case of hormone-dependent breast, uterus, and prostate cancers.<br>A decrease in the toxic effects related to chemotherapy. |
| **Antianemia** | Reduction of the blood platelets number.<br>Strengthen the hemoglobin level. |

| | |
|---|---|
| **Effects on bone tissue** | Inhibition of the formation of the osteoclastic cells. |
| **Antiallergic** | Inhibition of the histamine release by the mast cells (62% efficiency). |
| **Anti-depressant** | Raise mental capacity and strengthen the nervous system.<br>Treatment of mental and physical overtiredness, asthenia, and apathy.<br>Long term use induces gradual mood improvement and restoration of the desire of living. |

*In vitro* studies of bee pollen described the effect of this beehive extract on the inhibition of different cancer cell lines suchlike leukemia cells or prostate cancer PC-3 cells (Denisow & Denisow-Pietrzyk, 2016). The malignant tumor is made of abnormal cells that grew uncontrollably and went beyond their usual boundaries to overrun surrounding parts of the body and/or spread to further organs. This process is called metastasis which considered cancer's major cause of death (Who. int, 2019).

Bee pollen's extract bioactivity can vary according to the botanical and geographical origin and also the storing conditions (Mărgăoan et al., 2019). Consequently, an enormous diversity in the chemical composition of bee pollen can be revealed, including different phenolic and volatile compounds, vitamins, minerals, sterols, and lipids. Bee pollen possesses a wide variety of phenolic compounds which pharmacological activity in *in vitro* assays was demonstrated (Ares et al., 2018).

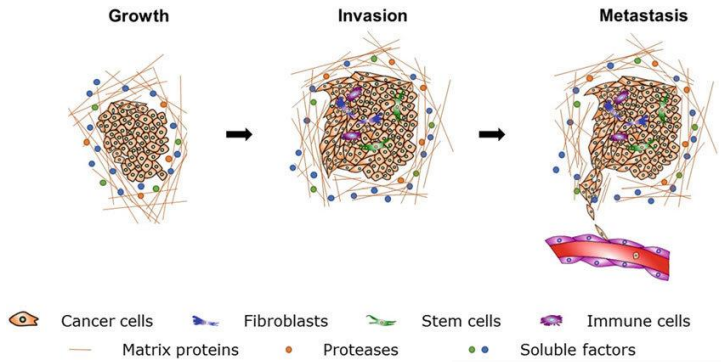


Figure 8: The progression of cancer tumor (Ghosh D., Dawson M.R., 2018)

The therapeutic effects of fatty acids have been demonstrated by the *in vitro* and *in vivo* studies on cancer cell development (Denisow & Denisow-Pietrzyk, 2016; Mărgăoan et al., 2019). Researchers showed a remarkable interest in unsaturated fatty acids (linoleic, linolenic, and arachidonic), particularly in α-linolenic acid. Also, some esters of fatty

acids have proved an antitumor activity in mice against Ehrlich ascites tumor (Denisow & Denisow-Pietrzyk, 2016). By promoting apoptosis, *Brassica campestris*'s bee pollen appeared to possess powerful cytotoxicity in human prostate cancer PC-3 cells by applying a steroid fraction of chloroform extract. Patients with benign prostatic hyperplasia (BPH) and others with prostate cancer in the initial stage showed improvement after the usage of a specific bee pollen such as rye, *Glycine max* (soy), or *Prunus mexicana* pollen extract. Yet, it must be noted that in these experiments the pollen usage was complementary to the chemotherapeutic treatment (Denisow & Denisow-Pietrzyk, 2016).

Consequently, recent studies examined the bioactive molecules in bee pollen concerning their potential chemotherapeutic capacity for treating cancer. Otherwise, these compounds must be considered as a synergistic agent with the anticancer drug as well as with the capacity for decreasing common adverse effects of the chemotherapy (Poleni et al., 2019).

# 3. Material and methods

## 3.1. Standards and reagents

Sodium phosphate ($Na_2HPO_4$), potassium phosphate ($KH_2PO_4$), potassium ferrocyanide ($C_6FeK_4N_6$), sodium persulfate ($Na_2S_2O_8$), trichloroacetic acid ($C_2HCl_3O_2$), formic acid ($CH_2O_2$), sulfuric acid ($H_2SO_4$), were purchased from Fisher Scientific (Pittsburgh, PA). Phenolic compounds standards (p-coumaric acid, quercetin, and chrysin), ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)], Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-Diphenyl-1-picrylhydrazyl) were obtained from Sigma Chemical Co (St Louis, MO, USA), and kaempferol was purchased from Extrasynthese (Genay, France). Folin-Ciocalteu's reagent, acetic acid ($CH_3COOH$), and sodium chloride (NaCl) were purchased from Panreac Applichem (Barcelona, Spain). Iron (III) chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), and naringenin were from Acros Organics (Pittsburgh, PA), sodium carbonate anhydrous ($Na_2CO_3$) were purchased from Labkem (Barcelona, Spain). HPLC-grade methanol, ethanol, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Water was treated in a Milli-Q water purification system (TGI pure system, Houston, TX, USA).

## 3.2. Pollen samples

Portuguese bee pollen samples were collected by local beekeepers during the spring/summer season of 2018 from two locations: Bragança and Nisa, Figure 9. The pollen samples were collected with pollen traps placed in front of the beehive entrance. After, the samples were cleaned of debris, they were kept in plastic bags at -18 °C until their delivery to the laboratory.

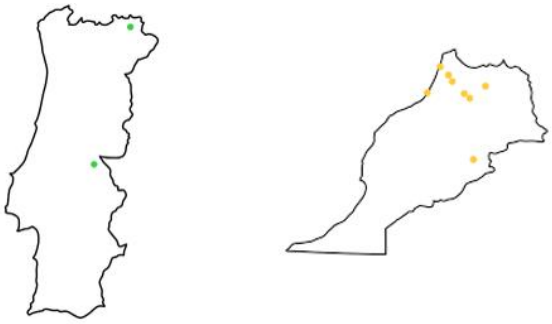

Figure 9: Place of origin of BP samples (a) Portugal's locations (b) Morocco's location

Finally, the pollen loads of each sample were manually separated according to color, shape, and size allowing us to get 2 samples from the Bragança location and 5

samples from the Nisa location. Also, eight Moroccan samples collected by local beekeepers, between 2015 and 2017 from different locations were analyzed, Table 3. Samples were stored in the freezer (-18°C), until further analysis.

Table3: Pollen samples origin

| Geographical location | Sample code | Visual color | Year |
|---|---|---|---|
| Nisa (PT) | P1 | beige | 2018 |
| | P2 | yellow | 2018 |
| | P3 | orange | 2018 |
| | P4 | light green | 2018 |
| | P5 | purple | 2018 |
| Bragança (PT) | P6 | Soft green | 2018 |
| | P7 | Dark yellow | 2018 |
| Larache (MR) | M1 | Light purple | 2015 |
| Khenichat (MR) | M2 | Yellow | 2016 |
| Hed Kourt (MR) | M3 | Orange | 2016 |
| Kenitra (MR) | M4 | Dark yellow | 2017 |
| Fez (MR) | M5 | Dark yellow | 2016 |
| Sefrou (MR) | M6 | Orange | 2017 |
| Arfoud (MR) | M7 | Light yellow | 2017 |
| Taza (MR) | M8 | Yellow | 2017 |

PT – Portugal; MR – Morocco

### 3.3. Palynological analysis

The palynological analysis was performed by the Vegetable Biology Laboratory at Vigo´s University. To 1 g of the previous pollen samples, 10 mL of distilled water was added. After mixing in a vortex, a 100 µL aliquot was placed in a slice, and after drying, one drop of glycerin jelly was added for permanent preparation. Pollen grains identification was performed by optical microscope with total magnification (400X and 1000X). A reference collection of the botanical laboratory of Vigo's University and different pollen morphology guides were used for the recognition of the pollen types.

### 3.4. Phenolic compounds analysis

#### 3.4.1. Extraction procedure

Aliquots of 2 g bee pollen samples were accurately grounded and weighed into a centrifuge tube and extracted with 15 mL 70% aqueous ethanol 70°C for 30 min, in a water bath at 100 RPM. The mixture was vacuum filtered, and the derived extract was stored at 5°C until analysis.

#### 3.4.2. Total phenolic and flavonoid content

##### 3.4.2.1.Total Phenolic content

Total phenolic compounds present in the hydroethanolic extracts were quantified spectrophotometrically through the Folin–Ciocalteu test following the protocol of Falcão *et al.* (2013b) with some modifications.

Briefly, 0.5 ml of the extract (5 mg/ml) was mixed with Folin–Ciocalteu's reagent (0.25 ml). After 3 min, 1 ml of a saturated $Na_2CO_3$ solution was added to the mixture and the volume was made up to 5 ml with distilled water. The solution was kept for 10 min at 70 °C and then cooled in the dark for 20 min. Thereafter, the mixture was centrifuged for 10 min at 5000 RPM and the absorbance was measured at 760 nm. Deionized water was used as the blank sample. A standard solution of gallic acid (0.001-0.25 mg/mL) was used for constructing the calibration curve ($y = 8.1477x + 0.0205$; $R^2 = 0.9998$). The total phenolic content (TPC) was expressed as mg of Gallic acid equivalents per g of bee pollen. The analysis was performed in triplicate.

##### 3.4.2.2. Total flavonoid content

The aluminum chloride method, previously described by Falcão *et al.*, 2013, was used to determine the flavonoid content of the samples. Briefly, 0.2 mL of sample (5mg/mL) was mixed with 0.2 mL of $AlCl_3$ solution (2% aluminium chloride in 5% acetic acid/methanol). After that, 2.8 mL of methanol with 5% acetic glacial acid was added. The mixture was then incubated for 30 min in the dark and the absorbance was measured at 415 nm. A mixture of sample and methanol (5% acetic glacial acid) was used as the blank sample. Standard solutions of quercetin (0.0016-0.05 mg/mL) was used for constructing the calibration curve ($y = 4.4625x + 0.0031$; $R^2 = 0.9992$). The total flavonoid content (TFC) was expressed as mg of quercetin equivalents per g of bee pollen. The experiment was run in triplicate.

3.4.3. Phenolic compounds profiling and quantification

The LC/DAD/ESI-$MS^n$ analyses were carried out using a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA) equipped with a diode-array detector and coupled to a mass detector. The used column was a 250 mm x 4mm id; 5 µm particle diameter, end-capped) Nuleosil C18 (Macherey-Nagel) and the temperature was maintained at 30°C. In terms of the mobile phase, it was composed of solution A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile), and both mixtures were previously degassed and filtered. The gradient elution of the mobile phase started with 80% A and 20% B until 10 min, 70% A and 30% B until 40 min, 40% A and 60% B until 60 min, 10% A and 90% B until 80 min then it returns to the initial conditions.

For analysis, 1mL/min was used as the flow rate, and after filtration through a 0.2 µm Nylon membrane (Whatman), 10 µl of each solution (133.33 mg/mL) was injected. The final spectra data were accumulated in the wavelength interval of 190-600 nm. The mass spectrometer was operated in the negative ion mode using Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, CA, USA) equipped with an ESI source. The source's voltage was 5kV, in addition to -20V and -65V for the capillary and the tube lens. The capillary's temperature was fixed to 325°C. Both sheath and auxiliary gas ($N_2$) flows were fixed to 50 and 10 (arbitrary units). Mass spectra were 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 the CID cell was set at 35 (arbitrary units). Data acquisition was carried out with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA).

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.Quantification was achieved using calibration curves for *p*-coumaric acid (0.00925-0.4 mg/mL; $y = 2.06 \times 10^7 x - 3.5 \times 10^5$; $R^2 = 0.973$), kaempferol (0.037-1.6 mg/mL; $y = 4.27 \times 10^6 x + 1.98 \times 10^5$; $R^2 = 0.983$), chrysin (0.0185-0.8 mg/mL; $y = 1.20 \times 10^7 x - 5.83 \times 10^4$; $R^2 = 0.999$), quercetin (0.037-1.6 mg/mL; $y = 3.9 \times 10^6 x + 4.65 \times 10^5$ ; $R^2$=0.937), naringenin (0.0185-0.8 mg/mL; $y = 7.85 \times 10^6 x - 3.04 \times 10^5$ ; $R^2$=0.978). When the standard was not available, the compound quantification was expressed in equivalent of the structurally closest compound. The results were expressed as mg/g of fresh pollen.

## 3.5. Volatiles analysis

### 3.5.1. Solid Phase Micro-Extraction

Preliminary optimization of the extraction time and the addition of saline solution led to optimum extraction conditions. To 2.5 g of ground bee pollen, introduced into a 25 mL glass vial, were added 2.5 mL of a 30 % sodium chloride solution and were all stirred until homogenization. The vial was sealed with a predrilled septum and placed in a thermostated bath at 50ºC. Headspace sampling was done using a manual SPME holder equipped with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) StableFlex fiber (Supelco, Bellefonte, PA, USA). A sampling of the volatile bee pollen compounds was done by inserting the fiber through the septum and exposing it to the headspace for 60 min with continuous stirring. The fiber was then retracted and transferred to the injector port of the gas chromatograph where the compounds were desorbed for 5 min.

### 3.5.2. Volatiles profiling and quantification

For the volatile analysis, the method described previously (Rodríguez-flores *et al.*, 2021) was followed with modifications. The GC-MS unit consisted of a Perkin Elmer system (GC Clarus® 580 GC module and Clarus® SQ 8 S MS module) gas chromatograph, equipped with DB-5 MS fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 6.1, Perkin Elmer, Shelton, CT, USA). The SPME fiber was desorbed at 250 ºC for 5 min. The oven temperature was programmed, 40-170°C, at 3°C.min$^{-1}$, subsequently at 25°C min$^{-1}$ up to 290°C, and then held isothermal for 15 min. The transfer line temperature was 280°C; ion source temperature, 230°C; carrier gas, helium, adjusted to a linear velocity of 40 cm.s$^{-1}$; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1s. Identifications were based on the comparison of the obtained spectra with those of the NIST mass spectral library and were confirmed using linear retention indices determined from the retention times of an n-alkane ($C_7$–$C_{36}$) (Supelco, Bellefonte, PA, USA) mixture analyzed under identical conditions, with a comparison with published data, and when possible with commercial standard compounds. Quantitation (average value for two replicates per sample) was carried out using relative values directly obtained from peak total ion current (TIC).

3.6. Biological properties assays

3.6.1. Antioxidant activity

Three different assays were used to assess the *in vitro* antioxidant potential of bee pollen extracts: scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, reducing power by ferricyanide/Prussian blue assay, and ABTS radical scavenging activity assay.

3.6.1.1. DPPH radical-scavenging activity assay

This method was performed using a 96 well Microplate. The reaction mixture consisted of 0.15mL of extract solutions with different concentrations, as shown in Table 4, and 0.15 mL ethanol containing DPPH radicals (0.024 mg/mL).

Table 4: Sample dilutions for the DPPH assay

| Initial concentration= 1 mg/mL | | |
|---|---|---|
| Dilutions | | |
| Sample volume (mL) | Added Ethanol 70% volume (mL) | Final concentration (mg/mL) |
| 5. $10^3$ | 0.145 | 0.034 |
| 10. $10^3$ | 0.14 | 0.071 |
| 20. $10^3$ | 0.13 | 0.15 |
| 30. $10^3$ | 0.12 | 0.25 |
| 40. $10^3$ | 0.11 | 0.36 |
| 50. $10^3$ | 0.10 | 0.5 |

The mixture was left to stand for 45 min in the dark, and the absorbance was recorded at 517 nm in the ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\%RSA = [(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100$$

where $A_{sample}$ is the absorbance of the solution containing the sample, and $A_{DPPH}$ is the absorbance of the DPPH solution. The findings were expressed in $EC_{50}$ ( sample concentration that gives half-maximum response) in mg/mL extract.

3.6.1.2. Reducing power assay

The capacity to reduce Fe (III) on the BP samples was evaluated according to the previously described method (Tomás *et al.*, 2017). Bee pollen extract (5 mg/mL) was

mixed with sodium phosphate buffer (pH=6.6, 1.25 mL). Potassium ferricyanide (1%, 1.25 mL) was added and the mixture was shaken and incubated at 50ºC for 20 min. Then, trichloroacetic acid (10%, 1.25 mL) was added. The mixture was centrifuged at 3000 RPM for 10 min and 1.25 mL was removed from the top to a new tube. Water (1.25 mL) and ferric chloride (0.1%, 0.25 mL) was added, and the absorbance was read at 700 nm in a spectrophotometer. Gallic acid was used as a standard for the calibration curve (y = 37.985x + 0.0067; $R^2$ = 0.9991) and the values were expressed as milligram of gallic acid equivalent per 1 g of sample.

#### 3.6.1.3. ABTS Radical Scavenging Activity Assay

The assay was performed following the previously described methods with modifications (Bicudo de Almeida-Muradian et al., 2020; Zhang, Liu and Lu, 2020). This assay was carried out to determine the ability of bee pollen samples to scavenge the ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] radical cation, using Trolox, as the standard for the calibration curve (y = 0.0001x+0.0005; $R^2$ = 0.9952). Stock $ABTS^+$ solution was prepared from 7 mM ABTS and 2.45 mM sodium persulfate in deionized water. The $ABTS^+$ solution was diluted with distilled water to obtain an absorbance of 0.700 (± 0.020) at 734 nm. Bee pollen extract (5mg/mL, 40 µL) was added to the diluted $ABTS^+$ solution (960 µL) and mixed immediately. The mixture was incubated for 10 min in the dark and the absorbance was determined at 734 nm. The Percentage of inhibition was calculated by the formula:

$$\%\ Inhibition = \frac{(1 - Asample)}{Acontrol} * 100$$

$A_{sample}$ was the absorbance of $ABTS^+$ solution with a sample, and $A_{control}$ is the absorbance of $ABTS^+$ solution without sample. The Trolox equivalent antioxidant capacity (TEAC) of the bee pollen samples (mM trolox/mg bee pollen extract) was calculated using the calibration curve as follows:

$$\text{TEAC (mM}\frac{\text{trolox}}{\text{mg}}\text{ BP extract)} = \frac{(\%\ \text{InhibitionSample} - \text{b})}{a} * \frac{aliquot\ volume\ (mL)}{bee\ pollen\ weight\ (mg)}$$

a and b are the slope and the intercept of the calibration curve.

### 3.6.2. Cytotoxic activity

To evaluate the cytotoxic activity of bee pollen extract with the Sulforhodamine B (SRB) colorimetric assay, 5 human tumor cell lines were used: AGS (stomach gastric adenocarcinoma), CaCo2 (Epithelial colorectal adenocarcinoma), HeLa (cervical

carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer) as well as hFOB (human Fetal Osteoblastic) a non-tumor cell line. The treatment solution was prepared from a 20 mg/mL hydroethanolic bee pollen extract which was freeze-dried and then diluted to various concentrations (125 µg/mL to 2000 µg/mL).

The cell lines subcultures were performed in RPMI-1640 medium enriched with 2mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FBS and kept in a humidified air incubator containing 5% CO2 at 37 °C. After 24h of incubation, the attached cells were then treated with different extract concentrations and incubated again for 48h. Afterward, the adherent cells were fixed with cold trichloroacetic acid (TCA 10%, 100 µL) and incubated at 4°C for 1h. Subsequently, the cells were washed with deionized water and dried. SRB solution (SRB 0.1% in 1% acetic acid, 100 µL) was added to each plate well and incubated for 30 min at room temperature. The unbound SRB was removed with 1% acetic acid and the plates were air-dried. The bound SRB was solubilized with Tris (10mM, 200 µL). To measure the absorbance at 540 nm, an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) was used (Abreu *et al.*, 2011). Elipticine was used as a positive control and the results were expressed as $GI_{50}$ values in µg/mL (sample concentration that inhibited 50% of the net cell growth).

### 3.7.Statistical analysis

The correlation between antioxidant activity and phenolic content was established by using Pearson's correlation coefficient measure. Differences in $P < 0.05$ were considered significant. The statistical analysis and handling of data was performed using Microsoft Excel and GraphPad Prism 8.4.

## 4. Results and discussion

### 4.1.Palynological analysis

The most important aspect that affects bee pollen commercial value is its botanical origin declaration. Table 5 reports the predominant pollen type and family, its pollen prevalence, and the classification of the samples according to the botanical source. Bee pollen is classified as monofloral when the major taxon is present at a relative frequency greater than 80% (Campos *et al.*, 2008). Samples from Portugal were collected from two different regions, Nisa and Bragança. Floral types were identified as dominant taxa (>80%), and consequently, considered monofloral (Campos *et al.*, 2008). Several botanical families were detected in Nisa samples such as Asteraceae, Oleaceae, Cistaceae, Rosaceae, and Boraginaceae in samples P1, P2, P3, P4, and P5 respectively. The samples P6 and P7, harvested in Bragança, belonged to Rosaceae and Fagaceae families and to *Rubus* sp. and *Castanea* sp. pollen type, respectively. Thus, only P4 and P6 had the same floral origin, with different geographical sources among the Portuguese samples. These results are associated with the fact that *Rubus* sp. and *Castanea* sp. plants are considered important honey vegetations in the region of Bragança (Estevinho et al., 2012). Also, (Dias et al., 2008) reported the presence of *Echium*, *Carduus*, and *Castanea* pollen in honey from Portugal.

Analyzing the relative frequency of the main pollen types in Moroccan pollen, samples M1, M4, M5, M7, and M8 were considered monofloral, belonging to *Coriandrum/Daucus* sp., *Olea europea*, *Raphanus* sp. and *Ononis spinosa/Astralagus* sp. respectively, Table 5. The sample M2, M3, and M6 samples were considered hetero floral although their pollen grains were from one plant family or genus which dominated with a relative frequency of over 45% (Mărgăoan *et al.*, 2014). M2 was mainly composed of the same botanical family, Brassicaceae, but different genus types, *Brassica* and *Sinapis.* Similarly, in M3 sample the most abundant family was Asteraceae with three different pollen types identified, namely, *Carduus*, *Taraxacum* and *Scorzonera.* M6 presented a quite different composition, with a relative frequency of *Helianthemum* sp. (Cistaceae) type above the 70% and the presence of minor amounts of *Anthemis* (Asteraceae) *and Lhytrum* (Lythraceae). Previous studies, confirmed that *Coriandrum* (Apiaceae), *Olea europea* (Oleaceae), *Tamarix* sp. (Tamaricaceae) *and Astralagus sp.* (Fabaceae) are families or species frequently cultivated, naturally present and visited by the honey bees in in the northwest of Morocco (Gharnit, Ennabili and El Hamdouni, 2000).

Table 5: The main floral component in bee pollen samples and their classification.

| Sample code | Dominant botanical family | Predominant pollen type (%) | Other pollen type present | Sample classification |
|---|---|---|---|---|
| P1 | Asteraceae | *Carduus* sp. (>80%) | *Echium sp., Asphodelus sp.* | monofloral |
| P2 | Oleaceae | *Ligustrum/Olea sp.* (>80%) | *Eucalyptus sp.* | monofloral |
| P3 | Cistaceae | *Cistaceae* (100%) | | monofloral |
| P4 | Rosaceae | *Rubus sp.* (100%) | | monofloral |
| P5 | Boraginaceae | *Echium sp.* (100%) | | monofloral |
| P6 | Rosaceae | *Rubus sp.* (100%) | | monofloral |
| P7 | Fagaceae | *Castanea sp.* (100%) | | monofloral |
| M1 | Apiaceae | *Coriandrum and Daucus sp.* (100%) | | monofloral |
| M2 | Brassicaceae | *Brassica sp.* (60%) | *Sinapis sp.* (30%), *Tamarix sp.* (<10%) | heterofloral |
| M3 | Asteraceae | *Carduus*/*Galactites sp.* (35%) | *Taraxacum sp.* (17%), *Scorzonera/Lactuca sp.* (8%), *Bellis sp.* (8%), *Olea europea* (8%), *Echium sp.* (6%), *Eucalyptus* (3%) | Heterofloral |
| M4 | Oleaceae | *Olea europaea* (>85%) | *Tamarix* sp. (<5%) | monofloral |
| M5 | Brassicaceae | *Raphanus sp.* (>80%) | *Sinapis sp*. (<10%) | monofloral |
| M6 | Cistaceae | *Helianthemum sp.* (>70%) | *Anthemis sp.* (<10%), *Lhytrum sp.* (<5%) | heterofloral |
| M7 | Fabaceae | *Ononis spinosa/Astralagus sp.* (>90%) | *Lhytrum sp., Quercus sp.* (<10%) | monofloral |
| M8 | Fabaceae | *Ononis spinosa/Astralagus sp.* (>90%) | *Lhytrum sp., Quercus sp.* (<10%) | monofloral |

### 4.2. Phenolic compounds analysis

#### 4.2.1. Total Phenolic Content

The bee pollen samples were screened for their total phenolic content and the results are resumed in table 6. The Folin-Ciocalteu assay is commonly used in clinical and nutritional studies to measure the total phenolic compounds content in biological samples (Lamuela-Raventós, 2017). The levels of total phenolics in bee pollen samples measured with this assay varied between the botanical and geographic origins. Concentrations of TPC of all the samples varied from 2.73 ± 0.6 to 35.76 ± 1.53 mg GAE/g . The Portuguese samples showed a higher average value with a TPC value of 14.63 ± 10.22 mg GAE/g , while the Moroccan samples presented an average value of 8.41 ± 4.39 mg GAE/g. The sample P7, chestnut pollen, contained the highest total phenolic content compared to all the other samples, with a value of 35.76 ± 1.53 mg GAE/g . The same high amount of total phenolics was observed in the *Castanea* pollen from Italy (Rocchetti et al., 2019). P4 and P6, which presented the same botanical origin from different geographical areas, *Rubus* sp., showed a total phenolic amount of 14.83 ± 0.29 and 19.33 ± 0.35 mg GAE/g respectively, which are in accordance with the previously described for this pollens (Rocchetti et al., 2019).

Table 6: Total phenolic and flavonoid content results

| Sample | Total Phenolic Content (mg GAE/g of BP) | Total Flavonoid Content (mg QE/g of BP) |
|---|---|---|
| P1 | 2.93 ± 0.81 | 0.71 ± 0.09 |
| P2 | 16.02 ± 0.75 | 4.80 ± 0.82 |
| P3 | 6.791 ± 1.01 | 2.45 ± 0.27 |
| P4 | 14.83 ± 0.29 | 1.20 ± 0.19 |
| P5 | 6.75 ± 1.13 | 2.3 ± 0.25 |
| P6 | 19.33 ± 0.35 | 0.73 ± 0.03 |
| P7 | 35.76 ± 1.53 | 3.09 ± 0.06 |
| M1 | 3.70± 0.12 | 1.21 ± 0.06 |
| M2 | 5.20 ± 0.43 | 0.82 ± 0.08 |
| M3 | 7.26 ± 0.47 | 0.47 ± 0.1 |
| M4 | 10.34 ± 0.65 | 2.08 ± 0.08 |
| M5 | 12.05 ± 0.29 | 1.38 ± 0.16 |
| M6 | 9.18 ± 0.23 | 3.19 ± 0.11 |
| M7 | 16.79 ± 1.1 | 1.73 ± 0.16 |
| M8 | 2.73 ± 0.6 | 1.12 ± 0.76 |

Among the Moroccan samples, the highest TPC was measured in the M7 sample, with a value of 16.79 ± 1.1 mg GAE/g, while the lowest TPC was quantified in the M8 sample, with a value of 2.73 ± 0.6 mg GAE/g.

Even though both the predominant pollen families were the same, they were harvested from different regions which can explain the difference found. The role of the free hydroxyl groups in reducing the Folin reagent is important for the capacity of monophenols, phenolic acids, aminophenols, and especially flavonoids in generating the blue chromophore (Veron L. Singleton, 1999). Alternatively, even though the Folin-Ciocalteu assay measures all phenolics, not all the compounds present in nature will respond similarly (Lamuela-Raventós, 2017). Depending on the solvent used for the extraction, the TPC values will be different. Romanian multifloral bee pollen extracted using methanol 80% (v/v) showed TPC values that ranged from 3.8 ± 0.03 mg GAE/g in Ericaceae *Calluna vulgaris* to 8.9 ± 0.03 mg GAE/g in Rosaceae *Prunus*, which were considered low compared to the amounts registered in this study (Rodica et al., 2013). Ethanol 70% (v/v) was used as a solvent in this study's phenolic extraction, yet, Silva *et al.*(2006) mentioned the importance of the ethyl acetate fraction of the BP's ethanolic extraction as it showed better recovery of phenolic compounds when determined with Folin-Ciocalteu method.

### 4.2.2. Total Flavonoid Content

The total flavonoid content in bee pollen varied from 0.47 ± 0.1 to 4.8 ± 0.82 mg QE/g. The highest content was quantified for Portuguese sample P2 (*Ligustrum/Olea* sp.), with a value of 4.8 ± 0.82 mg QE/g of BP and M6 (*Helianthemum* sp.) with a value of 3.19 ± 0.11 mg QE/g of BP, in the Moroccan ones. The average Portuguese bee pollen TFC obtained was 2.18 ± 1.36 and 1.5 ± 0.8 mg QE/g for the Moroccan samples. It was reported in a previous study, a high TFC in olive pollen, which belongs to the Oleaceae family as the P2 sample, when comparing to palm pollen (Feas et al., 2012). P7 showed a remarkable TFC value (3.09 ± 0.06 mg QE/g) which slightly matches the obtained value of three *Castanea* pollen samples from Portugal by Basuny *et al*., (2013). In previous studies on Portuguese bee pollen, the observed TFC varied between 4.5 and 7.1 mg QE/g (Morais et al., 2011; Feas et al., 2012) which is regarded as a great source of flavonoids since the daily intake estimated by Commenges et al. (2000) was 14.4 mg. Higher TFC values were detected by Singleton, (1999) in three pollen species collected by a stingless bee in Malaysia, where the mean value of all the obtained amounts was 24.27 ± 0.11 mg/g QE. Some samples contained a richer amount of flavones and flavonols than bee collected pollen from the region of Bahia, Brazil, which showed a maximum content of 2.5 mg QE/g (Menezes *et al.*, 2010).

### 4.2.3. Phenolic compounds profiling and quantification

The identification of the phenolic compounds in bee pollen samples from Portugal and Morocco was conducted using retention time, UV-VIS spectra, and mass fragmentation, in comparison with either commercial standards or with the analysis of the fragmentation pathways detected in $MS^n$ spectra and UV information available in the literature. ESI-MS in the negative ion mode was applied due to its elevated sensitivity in polyphenols analysis (Falcão et al., 2013). Quantification was based on linear calibration curves of the peak area versus standards in different concentrations. In all the fifteen samples, 96 compounds were identified, including 55 flavonols, 1 flavanone, 4 phenolic acids, and 36 phenylamides. Within the Portuguese bee pollen, 76 compounds were found, while in the Moroccan samples 50 compounds were identified. The peak characteristics, proposed compounds and quantification results of all the samples are presented in Table 9 and 10. Chromatographic profiles of the phenolic compounds from P1 and M3 are shown in Figure 10 and 12 respectively. The cumulative percentage phenolic composition of Portuguese and Morrocan BP are presented in Figure 11 and 13.

***Portuguese bee pollen***

Regarding the phenolics identification, P1, *Carduus* bee pollen, contained similar amounts of flavonoids and phenylamides. Only flavonol derivatives were detected in this sample, where two isorhamnetin glycosides were the most abundant ones, isorhamnetin-*O*-malonyl-pentosyl-hexoside (*m/z* 695) and isorhamnetin-*O*-malonyl-hexoside (*m/z* 563). Quercetin, methyl herbacetin, and kaempferol glycosides were also detected, such as quercetin-*O*-diglucoside (, *m/z* 625) as well as kaempferol-*O*-diglucoside ( *m/z* 609).

In Figure 10, Peak 5 was identified as a tri-substituted spermidine whereas peaks 6, 7 and 8 were identified as tetra-substituted spermidine isomers. Among all the identified compounds, two tetracoumaroyl spermine (*m/z* 785) isomers, detected at 31.8 and 34.0 min were the most abundant compounds. Although, P1's phenolic profile showed numerous phenylamides peaks, it presented a weak antioxidant capacity, especially in the DPPH scavenging assay results.

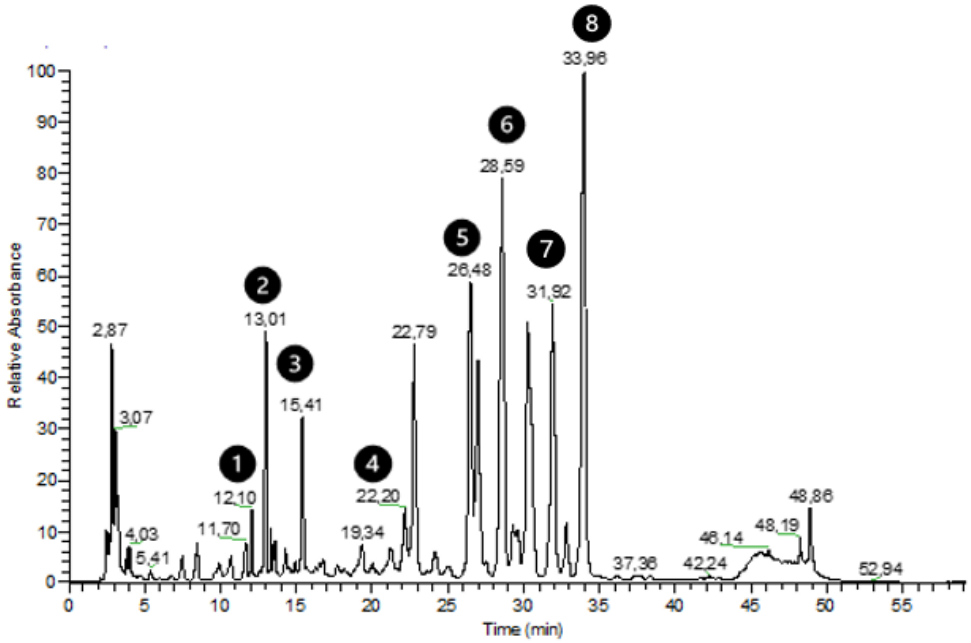


Figure 10: Chromatographic profile at 280 nm for P1 bee pollen: 1- Kaempferol-3-*O*-rutinoside; 2- Isorhamnetin-*O*-malonyl pentose-hexoside; 3- Isorhamnetin-*O*-malonyl-hexoside isomer; 4- $N^1$, $N^5$-di-*p*-coumaroyl-$N^{10}$-caffeoylspermidine; 5- $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine; 6- Tetracoumaroyl spermine isomer; 7- Tetracoumaroyl spermine isomer.

The sample P2, with a predominance of *Ligustrum* pollen, contained some flavonoids derivatives, such as kaempferol-*O*-diglucoside (*m/z* 609), kaempferol-3-*O*-rutinoside (*m/z* 593) and quercetin-*O*-diglucoside (*m/z* 625), which was the most abundant compound in this sample. Numerous phenylamide derivatives were detected such as feruloyl dicoumaroyl spermidine (*m/z* 612), $N^1$-feruloyl-$N^5$, $N^{10}$-dicaffeoylspermidine (*m/z* 644) and $N^1$, $N^5$-di-*p*-coumaroyl-$N^{10}$-caffeoylspermidine (*m/z* 598). This monofloral bee pollen was the only one to present a flavanone, naringenin (*m/z* 271) which was reported previously in the literature to be detected in Cistaceae *Cystus* and Fabaceae *Prosopis* (Jannesar et al., 2017).

Analyzing P3 sample, with a predominance of pollen from the Cistaceae family, 21 peaks of flavonoid glycosides were observed. Seven quercetin glycosides were identified, mainly quercetin-*O*-malonyl-hexoside (*m/z* 549) and quercetin-3-*O*-rhamnoside (*m/z* 447). In addition, laricitrin glycosides, only identified in this sample, and myricetin glycosides, already described in bee bread from Bragança, Portugal (Sobral *et al.*, 2017). Our results are in accordance with Maruyama *et al.*, (2010) findings as they

detected plenty of flavonoids and flavonoid glycosides especially quercetin, isorhamnetin and kaempferol glycosides in *Cistus* sp. bee pollen from Spain.

Both P4 and P6 samples had thesame floral origin, *Rubus sp.*, from the Rosaceae family, but were collected in different geographical regions. The two phenolic profiles were very similar, only differing in the quantities detected. Concerning flavonoids, methyl herbacetin glycosides were identified in both samples, in which methyl herbacetin-*O*-dihexoside (*m/z* 639) was the main flavonol identified with a value of 4.0±1.2 mg/g. Bee pollen P4 presented two additional flavonoid and phenylamide derivatives, namely, kaempferol-3-O-rutinoside (*m/z* 593) and tetracoumaroyl spermine (*m/z* 785), whencomparing to P6. Interestingly, the most abundant compound in both samples was the $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine (*m/z* 582) detected at an elution time of 26.6 min, with a higher amount quantified in P4 sample with a value of 11.9±0.3 mg/mL. High amounts of phenylamides are frequently found throughout the plant kingdom and particularly in pollen grains (Vogt, 2018). In previous studies, high phenylamides concentration in pollen grains was at first linked to plant fecundity until it was proved to be ubiquitous in pollen, depending their amount on the plant species, and their function (Elejalde-Palmett et al., 2015). P4 and P6 confirmed the hypothesis put by Campos *et al.*, (2003) as well as Ulusoy and Kolayli, (2014) that each plant species possess a profile of specific phenolic compounds. Nevertheless, it is necessary to consider some differences in bee pollen phenolic profiles from the same species due to the genotypic differences as well as the environmental conditions (soil quality, temperature, hydrological stress, and light intensity) (Ferreres et al., 2010; Sobral et al., 2017). Certainly, both samples similar composition assumed the role of the highest ABTS scavenging activity values among the Portuguese samples.

The *Echium* bee pollen, P5, showed few kaempferol glycosides with a prominent amount of kaempferol-3-*O*-rutinoside (*m/z* 593) and kaempferol-*O*-malonyl-hexosyl-deoxyhesoxide (*m/z* 679). An isorhamnetin glycoside, isorhamnetin-*O*-malonyl-pentosyl-hexoside (*m/z* 695) was also identified in a lower concentration.

Table 7: Retention time (Rt), wavelengths of maximum absorption (λmax), mass spectral data, proposed identification, and quantification of phenolic compounds in Portuguese samples

| Rt (min) | λmax (nm) | [M-H]- m/z | MSn (% base peak) | Proposed Compound | P1 | P2 | P3 | P4 | P5 | P6 | P7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 6.7 | 259, 356 | 625 | 317 (100) | Myricetin-3-*O*-rutinoside[a,l] | nd | nd | 0.7±0 | nd | nd | nd | nd |
| 7.5 | 257, 353 | 625 | 301 (100), 300 (90), 445 (82), 271 (15) | Quercetin-*O*-diglucoside[a,d] | 0.2±0 | 5.0±0.2 | nd | nd | nd | nd | nd |
| 8.2 | 258, 356 | 711 | 667 | Myricetin-*O*-malonylrutinoside[a,l] | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 8.5 | 271, 325sh, 353sh | 639 | 271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20) | Methyl herbacetin-*O*-dihexoside[a,e] | 0.2±0 | nd | nd | 4.3±0.1 | nd | 4.0±1.2 | nd |
| 9.2 | 258, 356 | 639 | 330 (100), 331 (47), 315 (21), 607 (32), 519 (17) | Laricitrin-*O*-hexosyl-deoxyhexoside[a,e] | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 9.3 | 265, 349 | 609 | 284 (40), 285 (98), 429 (100) | Kaempferol-*O*-dihexoside[a] | 0.2±0 | nd | nd | nd | nd | nd | nd |
| 9.6 | 256355 | 609 | 301 (44), 300 (100) | Quercetin-*O*-rutinoside[a,d] (isomer) | nd | nd | 0.4±0 | nd | nd | nd | nd |
| 9.7 | 257, 354 | 609 | 300 (100), 301(47) | Quercetin-3-*O*-rutinoside[a,h,d] | nd | 0.3±0 | 0.5±0 | nd | nd | nd | nd |
| 10.2 | 266, 348 | 609 | 285 (95), 429 (100), 447 (12) | Kaempferol-*O*-diglucoside[a,f] | nd | 0.6±0 | nd | nd | nd | nd | nd |
| 10.3 | 265, 349 | 609 | 284 (40), 285 (98), 429 (100) | Kaempferol-*O*-dihexoside[a,f] (isomer) | 0.2±0 | nd | nd | nd | nd | nd | nd |
| 10.5 | 254, 354 | 623 | 461 (100) | Isorhamnetin-*O*-hexosyl-*O*-deoxyhexoside[a,h] (isomer) | nd | nd | 0.2±0.0 | nd | nd | nd | nd |
| 10.5 | 259, 355 | 565 | 521 (100) | Myricetin-*O*-malonyl hexoside[a] | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 10.7 | 271, 331sh, 358sh | 725 | 681 (100) | Methyl herbacetin-malonyl-dihexoside[a] | 0.2±0 | nd | nd | 2.1±0 | nd | 1.9±0.5 | nd |
| 10.7 | 269, 347 | 623 | 299 (58), 300 (37), 314 (100), 315 (70), 459 (89), 608 (32) | Methylherbacetin-3-*O*-rutinoside[a,e] | 0.3±0 | nd | nd | nd | nd | nd | nd |
| 11.0 | 266, 348 | 593 | 447 (100), 431 (48), 285 (8) | Kaempferol-*O*-hexosyl-*O*-deoxyhexoside[a] | nd | nd | 0.3±0 | nd | nd | nd | nd |
| 11.6 | 254, 354 | 609 | 315 (100) | Isorhamnetin-*O*-pentosyl-hexoside[a,d] | 0.2±0 | nd | nd | nd | nd | nd | 0.6±0 |
| 11.8 | 256, 355 | 695 | 651 (100) | Quercetin-*O*-malonyl deoxyhexosyl-hexoside[a] | nd | nd | 0.2±0 | nd | nd | nd | 0.7±0 |
| 12.1 | 267, 347 | 593 | 284 (100), 285 (69) | Kaempferol-3-*O*-rutinoside[a,h,e] | 0.2±0 | 0.5±0 | 0.3±0 | 0.2±0 | 3±0.5 | nd | nd |
| 12.3 | 254, 354 | 623 | 314 (100), 315 (85), 459 (80) | Isorhamnetin-3-*O*-hexosyl-deoxyhexoside[a] | nd | nd | 0.2±0 | nd | nd | nd | 0.4±0 |
| 12.6 | 256, 354 | 463 | 301 (100) | Quercetin-3-*O*-glucoside[a,h] | nd | 0.3±0 | 0.2±0 | nd | nd | nd | 1±0 |
| 12.6 | 272, 327sh, 352sh | 709 | 665 | Methyl herbacetin-*O*-malonyl-hexosyl-deoxyhexoside[a] | nd | nd | nd | 0.2±0 | nd | 0.2±0 | nd |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12.8 | 253, 353 | 709 | 519 | Isorhamnetin-*O*-acetyl-hexoside[a,e] | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 13.0 | 255, 354 | 695 | 651 (100) | Isorhamnetin-*O*-malonyl pentose-hexoside[a] | 0.5±0 | nd | nd | nd | 0.2±0 | nd | nd |
| 13.3 | 256, 353 | 549 | 505 (100) | Quercetin-*O*-malonyl hexoside[a] | nd | nd | 0.8±0 | nd | nd | nd | nd |
| 13.4 | 266, 344 | 679 | 635 (100) | Kaempferol-*O*-malonylrutinoside[a] | 0.2±0 | 0.3±0 | 0.3±0 | nd | nd | nd | 0.2±0 |
| 13.4 | 266, 348 | 679 | 635 | Kaempferol-*O*-malonyl-hexosyl-deoxyhesoxide[a,l] | nd | nd | nd | nd | 1.4±0.2 | nd | nd |
| 13.5 | 272, 327sh, 352sh | 477 | 315 (100), 462 (50), 300 (15) | Methyl herbacetin-*O*-hexoside[a,e] | 0.2±0 | nd | nd | 0.2±0 | nd | 0.2±0 | nd |
| 13.5 | 253, 256 | 579 | 535 | Laricitrin-*O*-malonylhexoside[a] | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 13.9 | 254, 353 | 549 | 505 | Quercetin-*O*-malonyl hexoside[a] (isomer) | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 14.0 | 263, 347 | 447 | 285 (100), 284 (90) | Kaempferol-*O*-hexoside[a,d] | nd | nd | 0.2±0 | nd | 0.2±0 | nd | nd |
| 14.2 | 254, 347 | 447 | 301 (100) | Quercetin-3-*O*-rhamnoside[a,h] | nd | nd | 1±0 | nd | nd | nd | nd |
| 14.3 | 253.351 | 477 | 314 (100), 315 (53) | Isorhamnetin-3-*O*-glucoside[a,d] | 0.2±0 | nd | nd | nd | nd | nd | nd |
| 14.5 | 299, 308 | 436 | 316 (100) | di-*p*-coumaroylspermidine[a,j] | nd | 0.2±0 | nd | nd | nd | nd | 0.5±0 |
| 15.0 | 265, 344 | 417 | 284 (100), 285 (48) | Kaempferol-*O*-pentoside[a] | 0.2±0 | nd | nd | nd | nd | nd | nd |
| 15.0 | 295, 315 | 630 | 494 (86), 468 (100),358 (7) | *N¹*, *N⁵*, *N¹⁰*-tricaffeoylspermidine[a] | nd | 0.3±0 | nd | nd | nd | nd | nd |
| 15.4 | 254, 354 | 519 | 315 (100) | Isorhamnetin-*O*-hexoside[a,d] | nd | nd | 0.2±0 | nd | nd | nd | 0.4±0 |
| 15.4 | 255, 355 | 563 | 519 (100) | Isorhamnetin-*O*-(malonyl)hexoside[a,l] (isomer) | 0.4±0 | nd | 0.3±0 | nd | nd | nd | nd |
| 15.8 | 295, 316 | 630 | 468 (100), 494 (85), 495 (20) | *N¹⁰*, *N⁵*, *N¹⁰*-tricaffeoylspermidine[a] | nd | nd | nd | nd | nd | nd | 1.8±0.0 |
| 16.2 | 264, 341 | 431 | 285 (100) | Kaempferol-3-*O*-rhamnoside[a,h] | nd | nd | 0.2±0 | nd | nd | nd | 2±0 |
| 16.2 | 298, 318 | 630 | 494 (86), 468 (100),358 (7) | *N¹*, *N⁵*, *N¹⁰*-tricaffeoylspermidine[a] | nd | nd | nd | nd | nd | nd | 0.3±0.0 |
| 16.8 | 299.319 | 630 | 468 (100), 494 (84), 358 (7) | *N¹*, *N⁵*, *N¹⁰*-tricaffeoylspermidine[a] | nd | nd | nd | nd | nd | nd | 0.3±0 |
| 17.6 | 293, 315 | 644 | 468 (11), 482 (11), 508 (100) | *N¹*-feruloyl-*N⁵*, *N¹⁰*-dicaffeoylspermidin[a] (isomer1) | nd | 0.1±0 | nd | nd | nd | nd | 9±0.2 |
| 18.0 | 296, 315 | 614 | 494 (25), 478 (100), 452 (69), 358 (20) | *N¹*-*p*-coumaroyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a] (isomer1) | nd | 0.2±0 | nd | nd | nd | nd | 0.3±0 |
| 18.1 | 299, 310 | 598 | 462 (100), 452 (45), 478 (40) | *N¹*, *N⁵*-di-*p*-coumaroyl-*N¹⁰*-caffeoylspermidine[a,d] (isomer1) | nd | nd | nd | 0.2±0 | nd | 0.2±0 | 0.4±0 |
| 18.3 | 296, 315 | 614 | 494 (25), 478 (100), 452 (69), 358 (20) | *N¹*-*p*-coumaroyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a,d] (isomer2) | nd | 0.2±0 | nd | nd | nd | nd | nd |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18.7 | 296, 315 | 614 | 478 (100), 468 (20), 452 (68), 342(5) | *N¹-p*-coumaroyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine (isomer3) | nd | nd | nd | nd | nd | nd | 0.3±0 |
| 19.0 | 299, 310 | 598 | 462 (100), 452 (39), 478 (41) | *N¹*, *N⁵*-di-*p*-coumaroyl-*N¹⁰*-caffeoylspermidine[a,d] (isomer2) | nd | 0±0 | nd | 0.4±0 | nd | 0.4±0 | 0.7±0 |
| 19.3 | 298, 308 | 614 | 494 (24), 478 (100), 452 (78), 358 (18) | *N¹-p*-coumaroyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a] (isomer 4) | 0.2±0 | 0±0 | nd | 0.4±0.1 | nd | 0.3±0 | nd |
| 19.5 | 297, 319 | 644 | 468 (11), 482 (11), 508 (100) | *N¹*-feruloyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a] (isomer2) | nd | nd | nd | nd | nd | nd | 2±0 |
| 20.0 | 297, 319 | 644 | 508 (100), 482 (80), 468 (4) | *N¹*-feruloyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a] (isomer3) | nd | nd | nd | nd | nd | nd | 0.8±0 |
| 20.3 | 299, 310 | 598 | 478 (46), 462 (100), 452 (46), 342 (14) | *N¹*, *N⁵*-di-*p*-coumaroyl-*N¹⁰*-caffeoylspermidine[a,d] (isomer3) | nd | 0.2±0 | nd | 0.8±0 | nd | 0.7±0.2 | 1.3±0 |
| 21.2 | 290, 309 | 582 | 462 (100) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer3) | nd | nd | nd | 1.2±0 | nd | nd | nd |
| 21.3 | 268, 347 | 285 | 285 (100), 257 (13), 151 (20) | Kaempferol[a,m] | 0.3±0 | nd | nd | nd | nd | nd | 0.7±0 |
| 21.5 | 273 | 612 | 492 (100) | Feruloyl dicoumaroyl spermidine[a,j] (isomer 1) | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 22.2 | 297, 310 | 598 | 462 (100), 452 (43), 478 (42) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer6) | nd | nd | nd | nd | nd | 1.4±0.6 | nd |
| 22.2 | 299, 310 | 598 | 462 (100), 478 (39), 452 (34), 342 (14) | *N¹*, *N⁵*-di-*p*-coumaroyl-*N¹⁰*-caffeoylspermidine[a] (isomer4) | 0.3±0.1 | 0.4±0 | nd | 2.4±0.1 | nd | nd | 1.3±0.7 |
| 22.8 | 295, 310 | 582 | 462 (100), 436 (9), 342(7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer4) | nd | nd | nd | 2.6±0.3 | nd | 1.7±0.8 | nd |
| 23.1 | 291, 310 | 612 | 492 | Feruloyl dicoumaroyl spermidine[a,j] (isomer 2) | nd | nd | nd | nd | 0.2±0.1 | nd | 1.2±0.9 |
| 23.6 | 294, 310 | 628 | 508 (100), 492, 478, 466 | Polyamide derivative[a] | nd | nd | nd | nd | nd | nd | 1.4±1.1 |
| 24.0 | 291, 310 | 642 | 466 (16), 492 (78), 506 (57), 522 (100) | Diferuloyl coumaroyl spermidine[a,j] (isomer 1) | nd | nd | nd | nd | 0.2±0.02 | nd | nd |
| 24.1 | 295, 310 | 582 | 462 (100), 436 (9), 342 (6) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer1) | 0.3±0.2 | 0.2±0 | nd | 3.6±0 | nd | 2.1±1 | nd |
| 24.5 | 293, 310 | 612 | 492 | Feruloyl dicoumaroyl spermidine[a,j] (isomer 3) | nd | nd | nd | nd | 0.3±0.1 | nd | 1.6±0.6 |
| 25.0 | 295, 310 | 583 | 462 (100), 436 (9), 342 (7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer5) | nd | nd | nd | 1.4±0 | nd | 0.8±0.3 | nd |
| 25.4 | 291, 310 | 642 | 466 (16), 492 (78), 506 (57), 522 (100) | Diferuloyl coumaroyl spermidine[a,j] (isomer 2) | nd | nd | nd | nd | 0.2±0 | nd | 4.2±3.2 |
| 25.4 | 367 | 271 | 151 (100), 176 (10) | Naringenin[b] | nd | 0.7±0 | nd | nd | nd | nd | nd |
| 26.2 | 294, 310 | 612 | 492 | Feruloyl dicoumaroyl spermidine[a,j] (isomer 4) | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 26.5 | 295, 310 | 582 | 462 (100), 436 (10), 342 (7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer2) | 0.4±0.1 | 0.7±0 | 0.2±0 | 12.0±0.3 | 0.2±0.1 | 5.3±2.3 | nd |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 27.0 | 270 | 785 | 545 (14), 639 (13), 665 (100) | Tetracoumaroyl spermine[a,j] (isomer 1) | 0.5±0.2 | nd | nd | nd | nd | nd | 4±3.4 |
| 27.6 | 298, 310 | 612 | 492 | Feruloyl dicoumaroyl spermidine[a,j] (isomer 5) | nd | 0.2±0 | nd | 0.4±0 | 0.4±0.2 | 0.3±0 | nd |
| 28.6 | 276 | 785 | 665 (100) | Tetracoumaroyl spermine[a,j] (isomer 2) | 0.4±0.3 | 0.2±0 | 0.1±0 | nd | 0.3±0.1 | nd | nd |
| 29.3 | 280, 310sh | 785 | 665 | Tetracoumaroyl spermine[a,j] (isomer 3) | 0.2±0 | 0±0 | nd | nd | nd | nd | nd |
| 30.6 | 291 | 785 | 665 (100) | Tetracoumaroyl spermine[a,j] (isomer 4) | 0.4±0.2 | nd | nd | nd | nd | nd | nd |
| 32.8 | 294, 310 | 785 (100) | 665 (100), 639 (20), 545 (15) | Tetracoumaroyl spermine[a,j] (isomer 5) | 0.5±0.3 | nd | nd | nd | nd | nd | nd |
| 34.0 | 299, 310 | 785 | 545 (14), 639 (13), 665 (100) | Tetracoumaroyl spermine[a,j] (isomer 6) | 0.6±0.3 | 1.5±0.9 | nd | 0.2±0 | 0.2±0 | nd | nd |
| | | | | Total Phenolic Acids (mg/g) | nd | nd | nd | nd | nd | nd | nd |
| | | | | Total Flavonoids (mg/g) | 4.1±0.1 | 7.0±1.7 | 7.1±0.2 | 7.0±1.6 | 4.9±1.1 | 6.3±1.6 | 3.7±0.3 |
| | | | | Total Phenylamides (mg/g) | 4.9±0.12 | 6.3±0.3 | 0.6±0.1 | 25.5±3.1 | 2.6±0.1 | 14.1±1.4 | 31.5±2.0 |
| | | | | Total phenolic compounds (mg/g) | 9.0±0.1 | 15.1±0.9 | 7.6±0.2 | 32.9±2.7 | 7.5±0.7 | 20.9±1.4 | 36.2±1.7 |

Confirmed with: [a]MSn fragmentation; [b]standard; References: [d]El Ghouizi et al. (2020a); [e]Sobral et al. (2017) ; [f]Llorach et al. (2003); [h]Falcão et al. (2012); [j]Paupière et al.(2017); [l]Anjos et al. (2019); nd: not detected; Values expressed as mg of each compound/g sample.

Several phenylamides derivatives were detected in small amounts, mainly feruloyl dicoumaroyl spermidine (*m/z* 612) and its 5 isomers and tetracoumaroyl spermine (*m/z* 785) and its 2 isomers. Campos *et al.* (2003) identified phenolic compounds in an *Echium* BP sample from Portugal, mostly phenolic glycosides and phenolic acids, with a content of 5.9 ± 0.2 and 5.9 ± 0.2 mg/g pollen, respectively, which are not in accordance with the present results where two-thirds of the quantified phenolics were flavonoid glycosides. On the other hand, Moita *et al.* (2013) noted a significant quantity of flavonoid glycosides in Spanish *Echium* bee pollen, as well as Ferreres *et al.* (2010) who identified some kaempferol glycosides in Portuguese *Echium* bee pollen in accordance with our results.

Finally, the *Castanea* sp. bee pollen, P7, contained the highest number of phenolic compounds among all the samples. The flavonoids content is relatively important compared to the other samples but only isorhamnetin and kaempferol glycosides were present. In terms of phenylamides, a compilation of 17 substituted spermidines was identified: 6 isomers of tri-*p*-coumaroylspermidine (*m/z* 614), 3 isomers of $N^1$-feruloyl-$N^5$, $N^{10}$-dicaffeoylspermidine (*m/z* 644), 4 isomers of $N^1$-*p*-coumaroyl-$N^5$, $N^{10}$-dicaffeoylspermidine (*m/z* 614), and 4 isomers of $N^1$, $N^5$, $N^{10}$-tricaffeoylspermidine *(m/z* 630) which makes the total phenylamides content 30.1 ± 1.7 mg/g. A high DPPH, ABTS scavenging activity, reducing power capacity, TPC and TFC was shown by bee pollen P7 and which is in accordande with the identified phenolic compounds. Not only BP, authors, who worked on other *Castanea* plant components such as the shell (Vázquez *et al.*, 2008) and the leaves (Calliste *et al.*, 2005), explored their antioxidant aptitude to inhibit stable DPPH, superoxide anion, and hydroxyl radical measured using electrosonic spin resonance (Calliste *et al.*, 2005).

Phenolic compounds quantification in Portuguese samples revealed an important content, ranging from 7.48±0.71 mg/g for the sample P5 to 36.12±1.73 mg/g for P7. The cumulative percentage phenolic composition in the Portuguese BP samples is showed in Figure 10.Within the different phenolic compounds groups, only flavonoids and phenylamides were detected in the samples. Bee pollen from P3 sample, Cistaceae family, present a composition rich in flavonoids with a low quantity of phenylamides. On the contrary, bee pollen from P7 sample, Fabaceae family, were mostly composed by phenylamides and with a low quantity of flavonoids. Most of the samples, contained more

than 50% phenylamides. The lack of phenolic acids in the Portuguese samples did not show any impact on the antioxidant capacity.

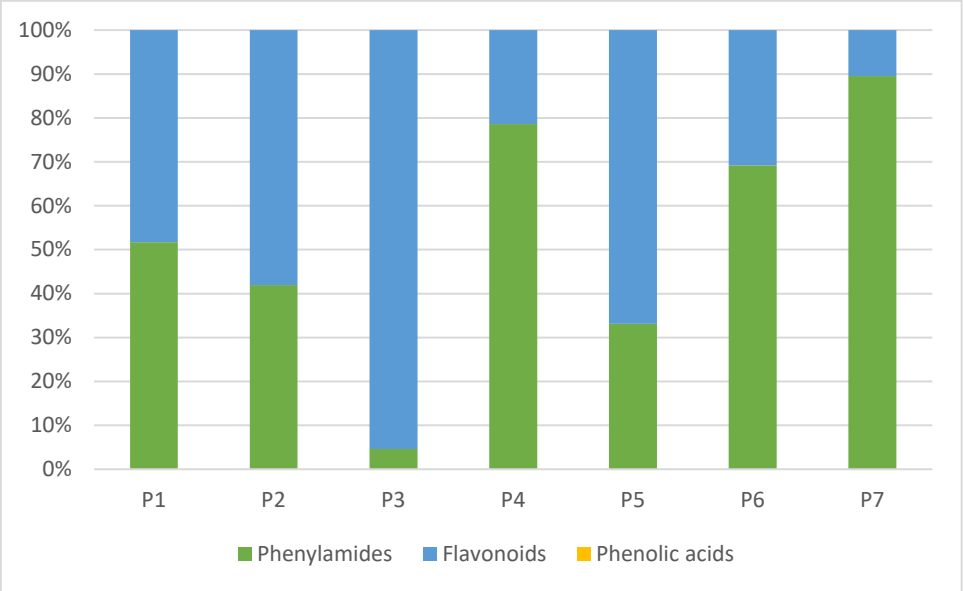


Figure 11: Cumulative percentage phenolic composition in the Portuguese BP samples

***Moroccan bee pollen***

Flavonoid glycosides predominated in the Moroccan samples, mainly quercetin, kaempferol, and isorhamnetin glycosides. Commonly, flavonoids are detected as *O*- or *C*-glycosides with sugar unites fixed to the hydroxyl or to one of the carbon atoms of the aglycone (De-Melo et al., 2018). The phenolic compounds were quantified ranging from 3.2±0.2 mg/g to 8.4±0.6 mg/g, showing M7 the highest content with 21.9±0.2 mg/g..

*Coriandrum sp.* bee pollen, sample M1, presented a unique profile containing *p*-coumaric acid (*m/z* 163), *p*-coumaric hexoside (*m/z* 325), and caffeic acid hexoside (*m/z* 341). Bee pollen samples from Italy belonging to the *Coriandrum* genus showed an important content of phenolic acids, especially *p*-coumaric acid derivatives and caffeic acid (Rocchetti et al., 2019). Regarding flavonoid compounds detected in M1, quercetin glycosides were the most abundant ones. Interestingly, quercetin-*O*-diglucoside (*m/z* 625) was detected, with a loss of O-hexose-hexose (-324 Da), in all the Moroccan samples in different amounts, from 0.5±0.2 mg/g in M3 to 3.3±0.1 mg/g in M4. Kaempferol glycosides were also identified, mostly kaempferol-*O*-rutinoside (peak, *m/z* 593), kaempferol-diglucoside (*m/z* 609), and kaempferol-*O*-rhamnoside (*m/z* 431).

The phenolic profile of M2, *Brassica*'s bee pollen, revealed as major peaks the flavonol glycosides quercetin-*O*-diglucoside (*m/z* 625) and kaempferol-*O*-diglucoside

(*m/z* 609). Rape bee pollen, which belong to the same genus, was mentioned in the literature due to its nutritional and therapeutic properties. Rape BP from china presented a quite different phenolic composition, principally, rutin, and benzoic acid. Other phenolics as quercetin, protocatechuic, and cinnamic acid were also identified (Zhang, Liu and Lu, 2020). However, we must consider the different environmental conditions, extraction methods, and analytical methods used in both studies which may certainly participate in the observed dissimilarity.

The heterofloral M3 bee pollen, containing *Taraxacum* plant's pollen, revealed the most important quantity of methyl herbacetin glycosides and isorhamnetin glycosides, such as isorhamnetin-*O*-malonyl-rutinoside (*m/z* 709) and isorhamnetin-*O*-malonyl-pentosyl-hexoside (*m/z* 695), as well as kaempferol glycosides like kaempferol-3-*O*-rhamnoside (*m/z* 431). Phenylamides, important metabolites that can be associated with diverse functions such as sporopollenin formation, pollen protection against UV or pollination were also detected (Elejalde-Palmett et al., 2015).

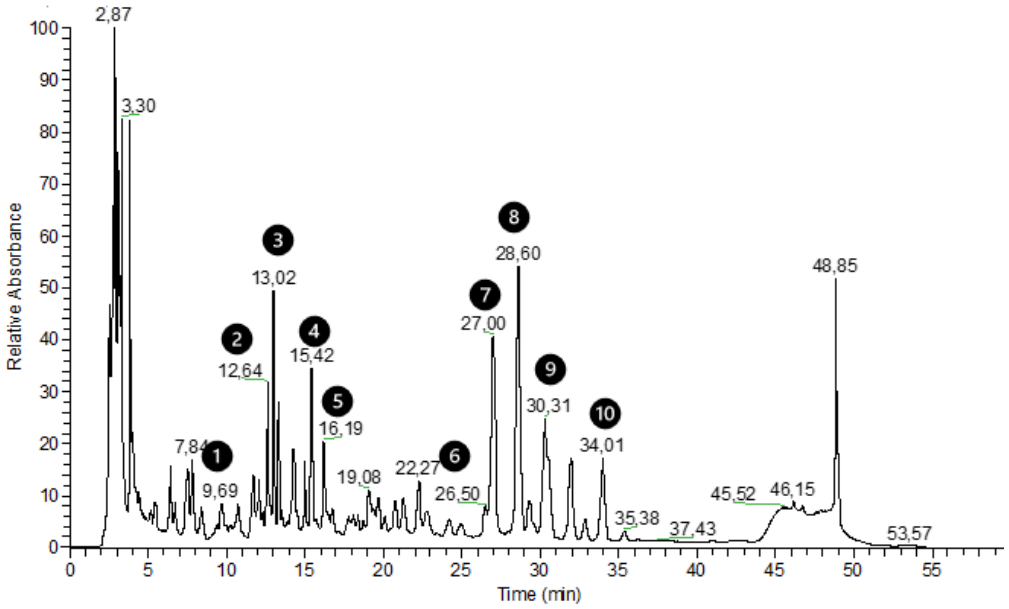


Figure 12: Chromatographic profile at 280 nm for M3 bee pollen: 1- Quercetin-3-*O*-rutinoside; 2- Isorhamnetin-*O*-malonyl-rutinoside; 3- Isorhamnetin-*O*-malonyl-pentose-hexoside; 4- Isorhamnetin-*O*-hexoside; 5- Kaempferol-3-*O*-rhamnoside; 6- $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine; 7- Tetracoumaroyl spermine isomer; 8- Tetracoumaroyl spermine isomer; 9- Tetracoumaroyl spermine isomer; 10- Tetracoumaroyl spermine isomer

Table 8: Retention time (Rt), wavelengths of maximum absorption (λmax), mass spectral data, proposed identification, and quantification of phenolic compounds in the Moroccan samples

| Rt (min) | λmax (nm) | [M-H]- m/z | MSn (% base peak) | Proposed Compound | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.0 | 290, 322 | 341 | 179 (100), 135 (2) | Caffeic acid hexoside[a,c] | 0.4±0 | nd | nd | nd | 0.1±0 | nd | nd | nd |
| 4.0 | 292, 322 | 179 | 179 | Caffeic acid[a] | nd | nd | nd | nd | nd | nd | nd | 0.2±0 |
| 4.1 | 300 | 325 | 163 (100) | *p*-Coumaric acid hexoside[a,b] | 0.2±0 | 0.2±0 | nd | nd | nd | nd | nd | nd |
| 6.7 | 259, 356 | 625 | 317 (100) | Myricetin-3-*O*-rutinoside[a,d,e] | nd | nd | 0.2±0 | nd | nd | 0.8±0 | nd | nd |
| 7.5 | 257, 353 | 625 | 301 (100), 300 (90), 445 (82), 271 (15) | Quercetin-*O*-diglucoside[a,d] | 1±0 | 2±0 | 0.2±0 | 3.3±0.1 | 1.1±0.1 | 0.5±0.2 | 0.7±0 | 0.4±0 |
| 8.1 | 258, 255 | 667 | 316 (100), 317 (20), 625 (29) | Myricetin-*O*-acetyl deoxyhexosyl-hexoside[a] | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 8.4 | 267, 347 | 639 | 315 (100), 477 (74), 300 (5) | Methylherbacetin-*O*-dihexoside[a,g] | nd | 0.2±0 | 0.2±0 | nd | nd | nd | nd | nd |
| 9.1 | 257, 357 | 479 | 317 (100) | Myricetin-*O*-hexoxide[a] | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 9.6 | 256, 355 | 609 | 301 (44), 300 (100) | Quercetin-*O*-rutinoside[a,d] (isomer) | nd | nd | nd | nd | nd | 0.5±0 | nd | nd |
| 9.7 | 256, 352 | 595 | 271 (11), 300 (100), 301 (56), 445 (37), 463 (25) | Quercetin-*O*-pentosyl-hexoside[a] | nd | nd | nd | nd | nd | nd | nd | 0.2±0 |
| 9.7 | 257, 354 | 609 | 300 (100), 301(47) | Quercetin-3-*O*-rutinoside[a,i,d] | nd | nd | 0.2±0 | 0.4±0.1 | 0.3±0.1 | 0.3±0.2 | nd | nd |
| 10.2 | 266, 348 | 609 | 285 (95), 429 (100), 447 (12) | Kaempferol-*O*-diglucoside[a,f] | nd | 0.8±0 | nd | nd | 0.8±0 | nd | 0.3±0 | 0.2±0 |
| 10.5 | 254, 353 | 623 | 477 (100), 461 (50), 315 (11) | Isorhamnetin-*O*-deoxyhexosyl-O-hexoside[a] | nd | nd | nd | 0.2±0 | nd | nd | nd | nd |
| 10.5 | 259, 355 | 565 | 521 (100) | Myricetin-*O*-malonyl hexoside[a] | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 10.7 | 269, 347 | 623 | 299 (58), 300 (37), 314 (100), 315 (70), 459 (89), 608 (32) | Methylherbacetin-3-*O*-rutinoside[a,g] | nd | nd | 0.2±0 | nd | nd | nd | nd | 0.2±0 |
| 11.0 | 266, 347 | 593 | 285 (8), 431 (50), 447 (100) | Kaempferol-*O*-deoxyhexosyl-*O*-hexoside[a] | nd | nd | nd | nd | 0.2±0. | 0.3±0 | nd | nd |
| 11.6 | 254, 354 | 609 | 315 (100) | Isorhamnetin-*O*-pentosyl-hexoside[a,d] | nd | nd | 0.2±0 | nd | nd | nd | 0.2±0 | 0.2±0 |
| 11.6 | 254, 354 | 609 | 315 (100) | Isorhamnetin-*O*-pentosyl-hexoside[a,d] (isomer) | nd | nd | nd | nd | nd | nd | nd | 0.2±0 |
| 11.6 | 310 | 163 | 118 | p-coumaric acid[a] | 0.2±0 | nd | nd | nd | nd | nd | nd | nd |
| 11.8 | 256, 355 | 695 | 651 (100) | Quercetin-*O*-malonyl deoxyhexosyl-hexoside[a] | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 12.1 | 267, 347 | 593 | 284 (100), 285 (69) | Kaempferol-3-*O*-rutinoside[a,h,e] | nd | nd | 0.2±0 | nd | 0.3±0.1 | 0.3±0 | 0.2±0 | 0.2±0 |
| 12.3 | 254, 354 | 623 | 314 (100), 315 (85), 459 (80) | Isorhamnetin-3-*O*-hexosyl-deoxyhexoside[a] | nd | nd | nd | nd | 0.8±0.2 | nd | nd | nd |
| 12.6 | 256, 354 | 463 | 301 (100) | Quercetin-3-*O*-glucoside[a,h] | nd | nd | nd | 0.4±0 | nd | 0.2±0 | nd | nd |
| 12.6 | 253, 352 | 709 | 665 (100) | Isorhamnetin-*O*-malonyl rutinoside[a,k] | nd | nd | 0.2±0 | nd | nd | nd | nd | nd |
| 13.0 | 255, 354 | 695 | 651 (100) | Isorhamnetin-*O*-malonyl pentose-hexoside[a] | nd | nd | 0.3±0 | nd | nd | nd | nd | nd |
| 13.3 | 256, 353 | 549 | 505 (100) | Quercetin-*O*-malonyl hexoside[a] | nd | nd | 0.2±0 | nd | nd | 0.8±0 | nd | nd |
| 13.5 | 254, 359 | 579 | 535 (100) | 3',4',5',3,5,6,7-heptaFlavones-*O*-malonyl hexoxide[a] | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 13.9 | 254, 355 | 549 | 505 (100) | Quercetin-*O*-acetylhexoside[a] (isomer) | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |
| 14.2 | 254, 347 | 447 | 301 (100) | Quercetin-3-*O*-rhamnoside[a] | 0.2±0 | nd | nd | nd | nd | 1.2±0 | nd | nd |
| 14.3 | 253.351 | 477 | 314 (100), 315 (53) | Isorhamnetin-3-*O*-glucoside[a,d] | nd | nd | 0.2±0 | nd | nd | nd | nd | 0.2±0 |
| 14.3 | 254, 354 | 519 | 315 (100) | Isorhamnetin-*O*-hexoside[a] (isomer) | nd | nd | nd | nd | nd | 0.2±0 | nd | nd |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15.0 | 265, 347 | 533 | 489 | Kaempferol-*O*-malonyl rutinoside[a] | nd | nd | 0.2±0 | nd | nd | 0.3±0 | nd | nd |
| 15.4 | 254, 354 | 519 | 315 (100) | Isorhamnetin-*O*-hexoside[a,i,e] | nd | nd | 0.3±0 | nd | nd | 0.4±0 | nd | nd |
| 16.2 | 264, 341 | 431 | 285 (100) | Kaempferol-3-*O*-rhamnoside[a,e] | 1.6±0 | nd | 0.2±0 | 0.4±0 | nd | nd | nd | 0.2±0 |
| 19.3 | 298, 308 | 614 | 494 (24), 478 (100), 452 (78), 358 (18) | *N¹*-*p*-coumaroyl-*N⁵*, *N¹⁰*-dicaffeoylspermidine[a,d] | nd | nd | nd | nd | 0.2±0 | 0.2±0 | 0.4±0.1 | nd |
| 21.2 | 290, 309 | 582 | 462 (100) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer3) | nd | nd | 0.1±0 | nd | 0.2±0 | nd | nd | nd |
| 21.3 | 268, 347 | 285 | 285 (100), 257 (13), 151 (20) | Kaempferol[b] | nd | nd | nd | nd | nd | 0.3±0 | 3±0 | 0.4±0 |
| 22.0 | 353, 370 | 315 | 300 (100) | Isorhamnetin[b] | nd | nd | nd | nd | nd | 0.2±0 | 2±0.8 | 0.2±0 |
| 22.2 | 299, 310 | 598 | 462 (100), 478 (39), 452 (34), 342 (14) | *N¹*, *N⁵*-di-*p*-coumaroyl-*N¹⁰*-caffeoylspermidine[a,d] (isomer4) | nd | nd | nd | nd | nd | nd | 0.5±0 | nd |
| 22.8 | 295, 310 | 582 | 462 (100), 436 (9), 342(7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer4) | nd | nd | nd | nd | 0.2±0 | 0.2±0 | 2.5±0.1 | 0.2±0 |
| 24.1 | 295, 310 | 582 | 462 (100), 436 (9), 342 (6) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer1) | nd | nd | nd | nd | 0.2±0 | 0.2±0 | 1.7±0 | 0.2±0 |
| 25.0 | 295, 310 | 583 | 462 (100), 436 (9), 342 (7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer5) | nd | nd | nd | nd | nd | nd | 0.6±0 | 0.2±0 |
| 26.5 | 295, 310 | 582 | 462 (100), 436 (10), 342 (7) | *N¹*, *N⁵*, *N¹⁰*-tri-*p*-coumaroylspermidine[a,d] (isomer2) | nd | nd | 0.2±0 | nd | 0.3±0 | 0.3±0 | 11±0.1 | 0.2±0 |
| 27.0 | 270 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer1) | nd | nd | 0.2±0 | nd | nd | 0.1±0 | nd | nd |
| 28.7 | 280, 307 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer2) | nd | nd | 0.2±0.1 | nd | nd | 0.2±0 | nd | nd |
| 29.3 | 277, 310 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer3) | nd | nd | 0.1±0 | nd | nd | nd | nd | nd |
| 30.3 | 289, 306 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer4) | nd | nd | 0.2±0 | nd | nd | nd | nd | nd |
| 31.9 | 293, 310 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer5) | nd | nd | 0.2±0 | nd | nd | nd | nd | nd |
| 34.0 | 299, 310 | 785 | 665 (100), 545 (14), 639 (13) | Tetracoumaroyl spermine (isomer6) | nd | nd | 0.2±0 | nd | nd | 0.2±0 | nd | nd |
| | | | | Total Phenolic acids (mg/g) | 0.78±0.1 | 0.2±0.0 | nd | nd | 0.1±0.0 | nd | nd | 0.2±0.0 |
| | | | | Total Flavonoids (mg/g) | 2.7±0.6 | 2.7±0.7 | 2.8±0.0 | 4.6±1.2 | 3.6±0.3 | 7.4±0.3 | 5.8±1.0 | 2.4±0.1 |
| | | | | Total Spermidine derivatives (mg/g) | 0±0 | 0±0 | 0.338±0.023 | 0±0 | 1.17±0.053 | 0.915±0.068 | 16.095±3.578 | 0.788±0.038 |
| | | | | Total phenolic compounds (mg/g) | 3.6±0.1 | 3.2±0.2 | 3.3±0.1 | 4.6±0.3 | 4.9±0.6 | 8.4±0.6 | 21.9±0.2 | 4.6±0.0 |

Confirmed with: [a]MSn fragmentation; [b]standard; References: [c]Kang et al. (2016); [d]El Ghouizi et al. (2020a); [e]Sobral et al. (2017) ; [f]Llorach et al. (2003); [g]Bakour et al. (2019); [h]Falcão et al. (2012) ; [i]Falcão et al. (2013) ; [k]Mihajlovic et al. (2015); nd: not detected; Values expressed as mg of each compound/g sample

The latter compounds family included $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine (*m/z* 582) and other tetra-substituted spermidine derivatives (*m/z* 785) displayed in Peaks 6,7, 8, 9 and 10, Figure 12. Besides *Taraxacum* pollen, the same also contained a percentage of 35% of *Carduus* pollen.

Previously, flavonol glycosides such as isorhamnetin-3-*O*-glucoside and kaempferol rutinoside were detected in the *Carduus* inflorescence, cultivated in Poland, which is in accordance with the reported results (Kozyra, Komsta and Wojtanowski, 2019). Compared to the P1, the *Carduus* BP from the Portuguese samples, the flavonol glycosides identified in both samples are almost similar, however, P1 presented a richer spermidines composition.

Regarding the M4 sample, with a predominance of *Olea europea* pollen, only flavonol glycosides were found, highlighting the major compound, quercetin-*O*-diglucoside. The abundance of the flavonoid derivatives in this sample, can explain the important antioxidant capacity that it showed in the DPPH assay compared to the other Moroccan samples.

*Raphanus* bee pollen, M5 sample, contained high quantities of flavonol glycosides, such as quercetin-*O*-diglucoside, isorhamnetin-3-*O*-glucoside, and kaempferol-diglucoside (*m/z* 609). This last compound presented an $MS^2$ fragment at *m/z* 285, which corresponded to kaempferol after the loss of *O*-hexosyl-hexoside moiety, most probably a sophoroside linked in the $C_3$ position of the aglycone. Previously, kaempferol-3-sophoroside was detected in *Raphanus raphanistrum* bee pollen from Portugal and New Zealand (M. Campos et al., 1997). Also, phenolic acid and phenylamides derivatives like caffeic acid hexoside (*m/z* 341) and $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine (*m/z* 598) isomers were found in small amounts.

*Helianthemum* pollen, M6 sample, presented the most important myricetin glycosides content within all Moroccan samples, such as myricetin-3-*O*-rutinoside (*m/z* 625) and myricetin-*O*-hexoxide (*m/z* 479) which showed the aglycone peak at (*m/z* 317). Furthermore, the HPLC quantified flavonoids, as well as the TFC of the M6 bee pollen, are both the highest values among the Moroccan samples. Also, $ABTS^+$ scavenging reaction stoichiometry for quercetin is exceptionally high as one quercetin molecule is capable to reduce 12 $ABTS^+$ molecules (Ilyasov et al., 2020). Consequently, M6 has higher content in quercetin glycosides among the Moroccan samples which can explain the notable antioxidant effect values in all the antioxidant assays.

Through the palynological analysis, M7 and M8 presented the same pollinic content, with a predominance of *Ononis Spinosa/Astralagus* pollen. However they showed different phenolic profiles, with M7 sample containing a higher amount of phenylamide derivatives (16.1±3.6 mg/g), which included $N^1$, $N^5$, $N^{10}$-tri-*p*-coumaroylspermidine isomers (*m/z* 582), $N^1$-*p*-coumaroyl-$N^5$, $N^{10}$-dicaffeoylspermidine (*m/z* 614), and $N^5$-di-*p*-coumaroyl-$N^{10}$-caffeoylspermidine (*m/z* 598), while flavonols were the main compounds in M8 sample. Also, M7 achieved the best $ABTS^+$ scavenging activity which can be justified by the phenylamides antioxidant capacity that has been reported to be higher than flavonoids capacity (Ilyasov *et al.*, 2020)

M8 *sample*, revealed a phenolic profile that resembles the Chinese *Astragalus* semen especially in the kaempferol glycosides content, with the detection of kaempferol-3-*O*-rutinoside (*m/z* 593) (Cavia-Saiz *et al.*, 2010). Figure 13 illustrates the cumulative phenolic groups present in each Moroccan sample, where the flavonoids were the main compounds with the exception of sample M7, from the Fabaceae family. This later sample contained more than 70% phenylamides whereas other samples like M4, from the Oleacea family, were marked by the absence of this compounds group. Phenolic acids were only detected in samples M1, M2, M5 and M8, with a maximum of more than 20% in sample M1, from the Apiaceae family.

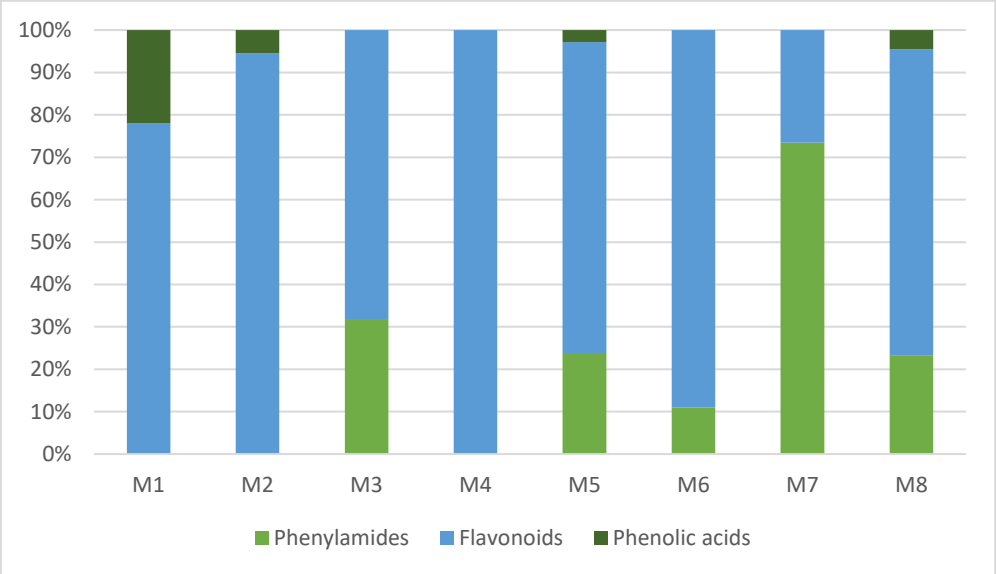


Figure 13: Cumulative percentage phenolic composition in the Moroccan BP samples

Overall, It was noted in the literature that cold weather may vary the phenolic metabolism in plants (Lattanzio, 2013) which can explain the difference between the

Portuguese and Morrocan phenolic quantities. It was assumed by scientists that each plant species owns a specific phenolic profile in both quantitative and qualitative terms (Maria Graça Campos et al., 2015). Accordingly, methyl herbacetin-*O*-malonyl-hexosyl-deoxyhexoside was detected only in the two *Rubus* samples, P4 and P6, which can be considered as a marker for this botanical origin.

### 4.3. Volatile compounds profiling and quantification

The SPME-GC-MS analysis in the current research served to study the relationships among the volatiles relative concentrations. Each compound quantity was obtained directly from the total ion current chromatogram (Total Ion Chromatogram, TIC) and expressed as a relative percentage. The identification of the detected compounds was performed using a commercial MS database (NIST 2011 mass spectral library). Linear retention indices (LRI) were calculated for each component detected. This allowed us to confirm the identification of each compound. For the calculation of the LRI indices, a mixture of n-alkanes (C7–C40) was used as reference series. A total number of 81 volatile compounds were identified in all the bee pollen samples, where 50 volatile compounds were identified in the Portuguese BP and 49 in the Moroccan ones. The volatile compounds list with the calculated LRI as well as relative concentration (R%) are displayed in Table 11 and 12. The Chromatographic profile of volatile compounds identified for bee polen P5 are presented in figure 14.

The identified components involve different classes of chemical compounds including terpenes (as thymol and eucalyptol), alcohols (as octanol and 1-hexanol), aldehydes (as nonanal and decanal), ketones (as sulcatone and jasmone), hydrocarbons (as nonadecane and hexadecane), esters (as ethyl decanoate and ethyl nonanoate) and, fatty acids (as nonanoic acid and caprylic acid). Bee pollen volatiles studies are infrequent in the literature, consequently, our samples composition may, by analogy, be compared to the honey's one.

Regarding the Portuguese samples, nonanal, heptadecane, nonadecane, hexanal and the two isomers of 3,5-octadien-2-one were the most abundant compounds, as shown for P5, figure 14. The cumulative percentage composition of the Portuguese samples is elucidated in figure 15, where the samples presented divergent compositions. P1 is the only sample that presented fatty acids among all the Portuguese samples.

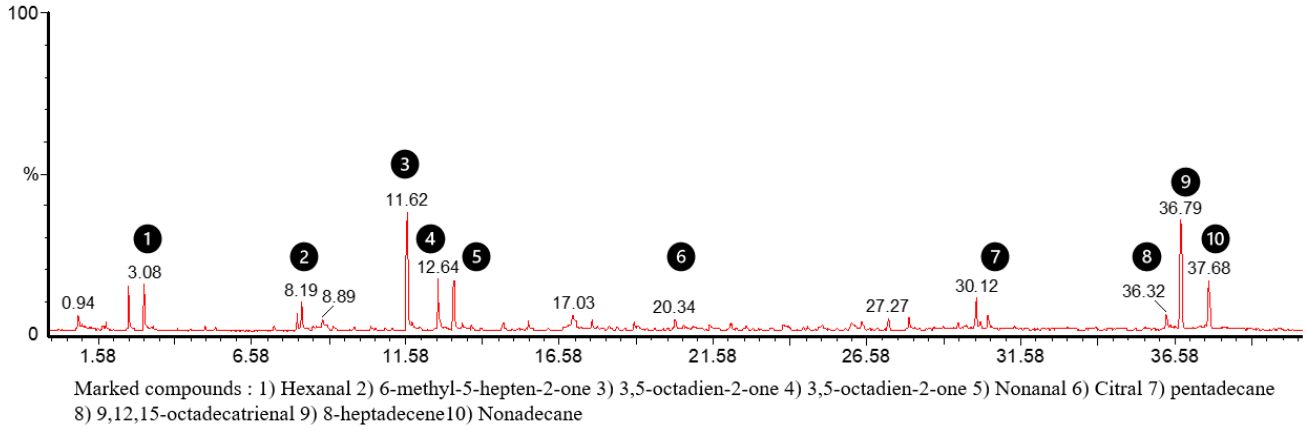


Figure 14: GC-MS chromatogram of P5 bee pollen sample

Esters were detected in all the Portuguese samples with different R%, as P1 contained less than 20%, while P7 displayed more than 80% esters of its composition and the rest of it was alcohols. Terpenes were only identified in P4, P5 and P6. Yet, the latter sample was the only bee pollen that contained more than 25% compounds which do not belong to the previously divided groups. Volatile Organic Compounds (VOCs) such as hexanal and 6-methyl-5-hepten-2-one were present in all the samples. Nonadecane was detected in all the samples except P3 and P7, while3,5-octadien-2-one appeared in 5 out of 7 volatile profiles. Certain compounds were only identified in a unique sample such as eucalyptol and linalyl authranilate in P6 and pentadecane in P5. Sample P7, with a predominance of chestnut BP, contained almost 69% nonanal, which is the highest R% mean value registered in all the samples. Also, octanal and octanol were present in remarkable R% too. The later sample contained the highest alcohols R% which can be derived from nectar or honey that bees used to form the BP pellets (Smith et al., 2002).

Concerning the Moroccan samples, hexanal, 3,5-octadien-2-one, 2,4-heptadienal and heptadecene were the most abundant compounds. Figure 16 showed the groups variance that are detected in the Moroccan samples where only M8 was the sample containing only 2 groups of VOCs, aldehydes and hydrocarbons. Octanoic acid was present in small R% in all the samples except the M8.

Table 9: Retention time (Rt), calculated LRI and quantification of volatile compounds in Portuguese samples

| Rt | Compound | LRI | P1 | P2 | P3 | P4 | P5 | P6 | P7 |
|---|---|---|---|---|---|---|---|---|---|
| 3.1 | Hexanal | 768 | 2.8±1.8 | 2.7±1.1 | 3.9±0.2 | 16.1±8.1 | 7.9±1 | 8.6±1.9 | 0.1±0 |
| 4.2 | 2-hexenal | 830 | 1.1±0.9 | nd | 0.8±0.1 | nd | nd | nd | nd |
| 5.0 | 1-hexanol | 849 | nd | nd | nd | nd | nd | 4.1±1.3 | 1.8±0.1 |
| 5.4 | Heptanal | 883 | 0.5±0.3 | 1.4±0.1 | 0.3987931 | nd | nd | 3.0±0.7 | 1.4±0.1 |
| 6.1 | Methyl hexanoate | 909 | 0.3±0.3 | 3±1.3 | 0.9±0 | nd | nd | nd | nd |
| 7.3 | (E,E)-2,4-heptadien-6-ynal | 943 | nd | nd | nd | nd | nd | nd | 0.5±0 |
| 7.3 | Benzaldehyde | 944 | 1.2±0.5 | nd | nd | nd | nd | nd | nd |
| 8.1 | 1-octen-3-ol | 965 | nd | nd | nd | nd | 1.8±0.1 | nd | 0.3±0.1 |
| 8.2 | 6-Methyl-5-hepten-2-one | 970 | 3.9±1.4 | 3.3±0.4 | 7.7±0.8 | 3.9±0.9 | 3.9±0.3 | 7.7±6.6 | 1.8±0.2 |
| 8.6 | 2,4-heptadienal | 998 | 6.6±2.1 | 3.9±0.1 | 0.5±0 | nd | nd | 2.4±0.7 | nd |
| 8.9 | Octanal | 988 | nd | nd | 2.1±1.4 | 4.3±1.2 | nd | 6.0±1.9 | 13.9±1.2 |
| 9.2 | 2,4-heptadienal (isomer) | 999 | nd | nd | 7.6±1.4 | 2.5±0.8 | nd | nd | nd |
| 9.9 | Eucalyptol | 1016 | nd | nd | nd | nd | nd | 2.1±0.3 | nd |
| 10.4 | 3,5,5-trimethyl-3-cyclohexen-1-one | 1026 | 0.9±0.1 | nd | nd | nd | nd | nd | nd |
| 11.7 | 3,5-octadien-2-one | 1057 | 10.5±3.4 | 5.2±0.8 | nd | 12.5±0.4 | 18.1±1.1 | 5.6±2.6 | nd |
| 11.8 | Octanol | 1059 | nd | nd | nd | nd | nd | nd | 8.2±1.3 |
| 12.6 | 3,5-octadien-2-one (isomer) | 1079 | nd | nd | 7.7±0.8 | 5.6±0.8 | 7.9±0.1 | nd | nd |
| 13.0 | Linalool | 1086 | nd | nd | nd | nd | nd | 5.6±0.9 | nd |
| 13.2 | Nonanal | 1091 | 2.7±1.9 | 2.5±2.4 | 11.7±0.8 | 7.8±2.6 | 7.3±0.2 | 0.2±0.2 | 67.2±1.8 |
| 13.4 | Phenylethylalcohol | 1097 | nd | nd | nd | nd | 1.2±0 | nd | nd |
| 13.7 | Isophorone | 1104 | nd | nd | nd | nd | 0.7±0 | nd | nd |
| 14.1 | Methyl octanoate | 1111 | 1.7±0.1 | 7.1±4.2 | 4.8±1.1 | nd | nd | nd | nd |
| 15.6 | Nonenal | 1144 | nd | nd | nd | nd | 1.8±0.2 | nd | nd |
| 16.2 | Nonanol | 1159 | nd | nd | nd | nd | nd | 4.5±1.7 | 2.6±0.1 |
| 17.3 | Ethyl octanoate | 1183 | 5.4±0.7 | 9.3±3.7 | nd | nd | nd | nd | nd |
| 17.7 | Decanal | 1191 | 0.7±0.2 | nd | nd | nd | 1.4±0.1 | 6.5±1.1 | nd |
| 19.0 | Cis-verbenol | 1219 | nd | nd | nd | 1.9±0 | 1.5±0 | nd | nd |
| 19.1 | Linalyl authranilate | 1223 | nd | nd | nd | nd | nd | 2.4±0.4 | nd |
| 20.3 | Citral | 1248 | nd | nd | nd | 3.3±0.4 | 2.2±0 | nd | nd |
| 21.4 | Thymol | 1273 | nd | nd | nd | 1.9±0.3 | 1.7±0.2 | nd | nd |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 22.6 | 3-(3-methyl-1-butenyl)cyclohexene | 1299 | 0.7±0.7 | nd | nd | nd | nd | nd | nd |
| 22.9 | Methyl decanoate | 1307 | 1.4±0.3 | 8.9±2.2 | nd | nd | nd | nd | nd |
| 25.3 | N-Decanoic acid | 1360 | 1.6±0 | nd | nd | nd | nd | nd | nd |
| 25.7 | Jasmone | 1370 | nd | 5.6±1.4 | nd | nd | nd | nd | nd |
| 25.9 | Ethyl hexadecanoate | 1377 | nd | nd | nd | 0.9±0.1 | nd | nd | nd |
| 26.0 | Ethyl decanoate | 1377 | nd | 2.9±0.4 | 4.8±0.2 | nd | nd | nd | nd |
| 26.4 | 1,3,5-trimethoxy-benzene | 1386 | nd | 6.9±4.3 | nd | nd | nd | nd | nd |
| 26.5 | Dodecanal | 1388 | 1.1±0.3 | nd | nd | nd | nd | nd | nd |
| 28.0 | Nerylacetone | 1423 | nd | nd | 16.5±0.7 | nd | 3.1±0.1 | nd | nd |
| 28.0 | 6,10-dimethyl-5,9-undecadien-2-one | 1425 | 6.4±1.5 | 8.1±8.1 | nd | nd | nd | 15.9±0.2 | nd |
| 29.6 | 2,6,10-trimethyltetradecane | 1462 | nd | nd | nd | nd | 0.6±0.4 | nd | nd |
| 30.1 | Hexadecane | 1474 | nd | nd | nd | 6.9±1.8 | 5.9±0.7 | nd | nd |
| 31.2 | Methyl dodecanoate | 1500 | 4.4±0.5 | 12.4±5.5 | nd | nd | nd | nd | nd |
| 33.2 | 7-hexadecene | 1551 | nd | nd | 4.5±0.7 | nd | nd | nd | nd |
| 36.3 | 9,12,15-octadecatrienal | 1642 | nd | 17±0.2 | nd | 0.4±0 | 2.1±0.1 | nd | nd |
| 36.4 | 9,12,15-octadecatrien-1-ol | 1634 | 2.7±0.9 | nd | nd | nd | nd | nd | nd |
| 36.5 | Octadecyne | 1636 | nd | nd | 4.3±0.3 | nd | nd | nd | nd |
| 37.0 | 8-heptadecene | 1649 | 36.8±12 | nd | nd | 21.7±2 | 21.7±1 | 10.9±5.1 | nd |
| 37.8 | Nonadecane | 1671 | 6.4±3 | nd | 22.1±1.3 | 10.4±0.7 | 9.1±0.8 | 14.5±2.1 | nd |

Rt: Retention time (min); LRI: Linear Retention Index determined on a DB-5 MS fused silica column relative to a series of n-alkanes ($C_7$-$C_{36}$); R%: Relative Percentage; nd: not detected.

Other compounds such as hexanal, 3,5-octadien-2-one and ethyl decanoate were present in 5 out of 8 samples with different R%. Other VOCs were detected in only one sample, for instance, 2,5-dimethyl-pyrazine in M1, geranyl vinyl ether in M3, anisaldehyde in M4, cis-β-terpineol in M5, ethyl hexanoate in M6, methyl octanoate in M7 and heptanal in M8. Sample M2, with predominance in *Brassica* BP, is the only sample that did not present any unique compound compared to the remainders.

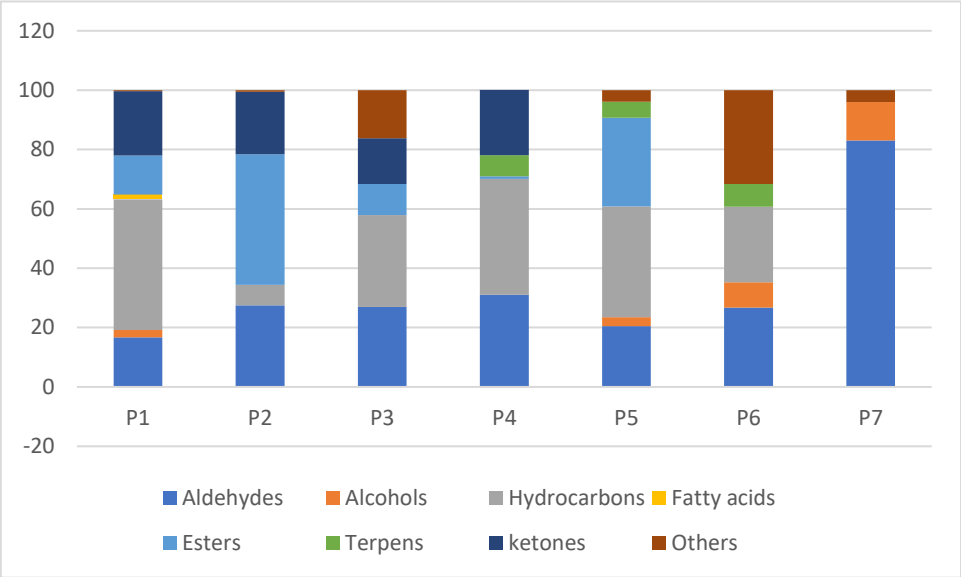


Figure 15: Cumulative percentage of volatiles' composition in Portuguese samples

Floral markers of some honey samples mentioned in the literature were detected in some of our samples. Nonanoic acid was reported as eucalyptus honey's floral marker from Italy (Piasenzotto et al., 2003). In addition, Radovic *et al.*, (2001) assumed nonanal, nonanol and nonanoic acid as eucalyptus honey' marker. However, nonanoic acid and nonanal were detected in M7, *Onis spinosa* BP, in addition to methyl nonanoate, ethyl nonanoate and methyl 8-methylnonanoate. Rape honey from Poland, belongs to the *Brassica* genus, contained p-cymene as specific floral marker which was absent in the M2 (Wardencki *et. al*, 2009). Sunflower honey from Italy, belongs to the Asteraceae family as P1 and M3, contained an important amount of aldehydes such as octanal, nonanal and decanal. Interestingly, P1 presented numerous aldehyde compounds such as hexanal, hexenal, heptanal, heptadienal, nonanal, decanal and dodecanal. Also, Radovic *et al.* (2001) remarked both linalool oxide and heptanal as typical marker compounds for Fabaceae *Acacia* honey from European countries, yet, linalool and heptanal were both detected in P6, Rosaceae *Rubus*. Though, Wardencki et *al.* (2009) mentioned hexanal as

a floral marker for *Acacia*' honey, where Manyi-Loh, Ndip and Clarke, (2011) declared that markers are used to discriminate honey's floral origins, consequently, they must be plant-derived molecules or their metabolites such as terpenes and benzene derivatives. Accordingly, in our samples it was possible to detected terpenes like caryophyllene, thymol, eucalyptol, among others, which can be considered as potential floral markers.

Radovic *et al.,* (2001) also reported 2-methyldihydrofuranone or methylbenzyl alcohol or both 3-hexen-1-ol and dimethylstyrene as markers for chestnut honey, however, P7 represented only one similar compound, 1-hexanol. On the other hand Bonaga *et al*., (1986) suggested 3-aminoacetophenone as special marker and main component of the *Castanea* honey's volatiles fraction, which was not identified in P7.

Lilac aldehyde and lilac alcohol were proposed to be some of the selected compounds characteristic of citrus honey, but identified in our study in the samples P1, *Carduus* BP. Kaškonienė *et al.,* (2015) notified about the misinterpretation of volatiles analysis that results, especially when low concentrated VOCs are originated from co-existing minor floral particles. Moreover, Santos-Buelga and González-Paramás, (2017) reported that volatile compounds may also serve as geographical discriminators of honey samples as some typical patterns exist for each country. Radovic *et al.,* (2001) suggested that some samples from The Netherlands, Portugal and Spain contained marker compounds where their presence or absence confirms the geographical origin. Concerning Portugal, 2,2,6-trimethylcyclohexanone, ethyl-2-hydroxypropanoate and 3-hexenylformate are possible marker compounds. Interestingly, 3,5,5-trimethyl-3-cyclohexen-1-one was detected in P1 which differs in the methyl groups positions and the added double bond in the cyclohexene ring compared to 2,2,6-trimethylcyclohexanone. Besides, Radovic *et al.,* (2001) proposed 1-octen-3-ol and 2,4-cycloheptadien-1-one-2,6,6-trimethyl as geographical markers of Spain. P5 and P7 were harvested from Bragança and Nisa which locations are near Spain land borders, and both contained 1-octen-3-ol.

Previous studies about volatile and semi-volatile organic compounds secreted by bees glands reported (E)-citral as an orientation pheromone secreted by the worker bees' Nasonov gland. The same compound was detected in both P4 and P5 (Smith et al., 2002). Also, nonanal was detected in worker bees' pheromone samples as well as

Table 10: Retention time (Rt), calculated LRI and quantification of volatile compounds in Moroccan samples

| Rt | Compound | LRI | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2.6 | 2-propenylidene-cyclobutene | 730 | nd | nd | nd | nd | 4.4±2 | nd | nd | 5.1±2.5 |
| 3.1 | Hexanal | 768 | 5.3±3.4 | 14.9±5.3 | nd | nd | nd | 13.5±0.4 | 21.0±1.6 | 66.2±1.6 |
| 4.2 | 2-hexenal | 830 | 2.2±1.7 | nd | nd | nd | nd | nd | 5.5±2.3 | 9.5±2.3 |
| 4.9 | Isothiocyanato-cyclopropane | 460 | nd | nd | nd | nd | 3.8±0.9 | nd | nd | nd |
| 5.4 | Heptanal | 883 | nd | nd | nd | nd | nd | nd | nd | 6.6±4.3 |
| 5.8 | 2,5-dimethyl-pyrazine | 901 | 3.9±1.3 | nd | nd | nd | nd | nd | nd | nd |
| 6.9 | 1,2-cyclopentanedione | 933 | nd | nd | nd | 1.4±1.3 | nd | nd | nd | nd |
| 8.3 | 2,4-heptadienal | 998 | 8.5±3.5 | 33.58±12.10 | nd | nd | nd | nd | 11.5±5.1 | nd |
| 8.8 | Ethyl hexanoate | 999 | nd | nd | nd | nd | nd | 5.7±3.8 | nd | nd |
| 8.9 | Octanal | 890 | 16.6±0.9 | nd | nd | nd | nd | nd | nd | nd |
| 9.3 | 2,4-heptadienal (isomer) | 631 | 9.2±0.3 | nd | nd | nd | nd | nd | nd | nd |
| 9.6 | Hexanoic acid | 1031 | nd | nd | 26.2±7.3 | nd | 21.3±7.2 | nd | nd | nd |
| 9.9 | Eucalyptol | 1016 | nd | 10.6±1.8 | nd | nd | nd | 7.1±0 | nd | nd |
| 11.7 | 3,5-octadien-2-one | 1057 | 22.9±1.5 | 27.5±27.1 | nd | nd | 2.7±1.1 | 13.9±5.2 | 23.9±2.4 | nd |
| 11.9 | 2,6,6-trimethylbicyclo[3.1.1]hept-3-ylamine | 1160 | 5.9±0.7 | nd | nd | nd | nd | nd | nd | nd |
| 12.7 | 3,5-octadien-2-one (isomer) | 1079 | 12.7±3.6 | nd | nd | nd | nd | nd | 25.6±0.7 | nd |
| 13.2 | Nonanal | 1091 | 1.9±1.6 | nd | nd | 2.7±0.6 | nd | nd | 1.7±0.6 | 5.8±3.1 |
| 13.2 | Cis-β-terpiniol | 1193 | nd | nd | nd | nd | 8.6±0.7 | nd | nd | nd |
| 14.1 | Methyl octanoate | 1111 | nd | nd | nd | nd | nd | nd | 1.5±0.8 | nd |
| 15.1 | Lilac aldehyde D | 1138 | 1.4±0.1 | nd | nd | nd | nd | nd | nd | nd |
| 15.3 | Pyranone | 951 | nd | nd | nd | nd | nd | nd | nd | nd |
| 15.4 | 2,6-nonadienal | 1142 | nd | nd | nd | nd | nd | nd | nd | 3.5±2.7 |
| 15.7 | Isopinocarveol | 1148 | nd | nd | nd | nd | nd | nd | 1.3±0.7 | nd |
| 17.3 | Octanoic acid | 1184 | 4.3±0.0 | 7.5±5.9 | 4±3.7 | 10.6±1 | 7.8±1.7 | 8.7±4.2 | 1.9±1.7 | nd |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 17.5 | Ethyl octanoate | 1183 | nd | 5.9±1.9 | 19.3±1.4 | 9.1±5.7 | nd | nd | 1.2±0.4 | nd |
| 17.8 | Lilac alcohol D | 1195 | 3.8±2.6 | nd | nd | nd | nd | nd | nd | nd |
| 18.1 | B-cyclocitral | 1201 | nd | nd | nd | nd | 2.8±0.4 | nd | nd | nd |
| 18.6 | Methyl 7-hexanoate | 1212 | nd | nd | nd | nd | nd | 1.6±0.4 | nd | nd |
| 18.7 | Methyl nonanoate | 1213 | nd | nd | nd | nd | nd | nd | 1.5±1.1 | nd |
| 19.8 | Anis aldehyde | 1238 | nd | nd | nd | 8.8±8.4 | nd | nd | nd | nd |
| 19.9 | Geranyl vinyl ether | 1240 | nd | nd | 0.7±0.6 | nd | nd | nd | nd | nd |
| 21.1 | 3-cyclohex-1-enyl-prop-2-enal | 1269 | 1.3±0.9 | nd | nd | nd | nd | nd | nd | nd |
| 21.3 | 2-methyl-1-nonene-3-yne | 1185 | nd | nd | nd | nd | nd | nd | nd | 3.3±0.3 |
| 21.9 | Ethyl nonanoate | 1285 | nd | nd | 4±0.9 | 6.9±1.7 | 7.7±2 | nd | 1.2±0.5 | nd |
| 22.0 | Nonanoic acid | 1287 | nd | nd | nd | nd | 8.6±1.9 | nd | 1.6±0.2 | nd |
| 23.3 | Methyl 8-methyl-nonanoate | 1315 | nd | nd | nd | nd | nd | nd | 0.7±0.4 | nd |
| 25.9 | 3-methyl-2-pent-2-enyl-cyclopent-2-enone | 1376 | nd | nd | nd | 5.4±2.5 | nd | nd | nd | nd |
| 26.3 | Ethyl decanoate | 1377 | nd | nd | nd | nd | nd | 13.2±2.3 | nd | nd |
| 26.3 | Methyl octanoate | 1385 | nd | nd | 11.5±3 | 26.8±4.2 | nd | 12±1.3 | nd | nd |
| 26.7 | Caryophyllene | 1394 | nd | nd | nd | 7.6±0.3 | nd | nd | nd | nd |
| 27.4 | Decanoic acid | 1411 | nd | nd | nd | nd | nd | 24.4±14.2 | nd | nd |
| 28.4 | 6,10-dimethyl-5,9-undecadien-2-one | 1425 | nd | nd | nd | 18.5±7.1 | 17.3±2.2 | nd | nd | nd |
| 28.5 | 4,6-dimethyl-(Z)-5,9-undecadien-2-one | 1261 | nd | nd | 7.5±0.2 | nd | nd | nd | nd | nd |
| 29.6 | B-ionone | 1399 | nd | nd | nd | nd | 1.7±1.3 | nd | nd | nd |
| 29.8 | B-ionone epoxide | 1466 | nd | nd | nd | nd | 11.7±0.2 | nd | nd | nd |
| 31.8 | 10-methyl-methyl undecanoate | 1400 | nd | nd | 1.5±1.5 | nd | nd | nd | nd | nd |
| 34.8 | Ethyl decanoate (isomer) | 1032 | nd | nd | 21.5±5.1 | 2.2±2 | nd | nd | nd | nd |
| 34.9 | Ethyl dodecanoate | 2161 | nd | nd | nd | nd | 5.5±1.8 | nd | nd | nd |
| 36.0 | 5-(1-piperidyl)-furan-2-carboxaldehyde | 1744 | nd | nd | 3.7±1.5 | nd | nd | nd | nd | nd |

Rt: Retention time (min) ; LRI: Linear Retention Index determined on a DB-5 MS fused silica column relative to a series of n-alkanes ($C_7$-$C_{36}$); R%: Relative Percentage; nd: not detected.

octanol, hexanol, benzaldehyde and caryophyllene which were reported in our study either (Keeling et al., 2004; Schiestl, 2010).

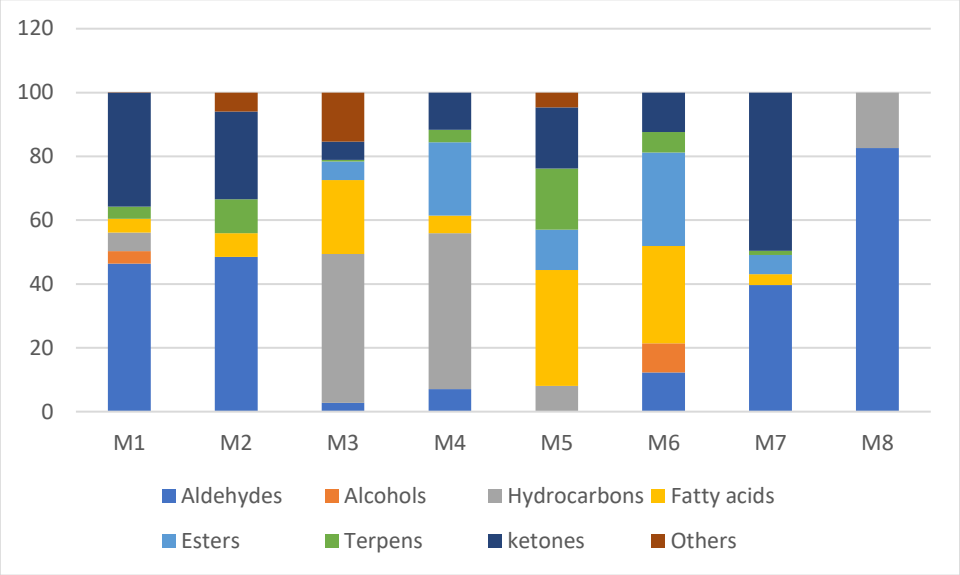


Figure 16: Cumulative percentage of volatiles' composition in Moroccan samples

### 4.4. Biological properties assays

#### 4.4.1. Antioxidant activity

The antioxidant activity of the extracts depend mainly on the methodology used. Indeed, the antioxidant capacity of any compound is usually dissimilar according to the applied assay due to the different interactions of the oxidant and the oxidizable substrate used in the measurement (Marghitas, 2014; Mohdaly *et al.*, 2015)

As a result, it is crucial to compare various analytical methods with the purpose to understand the biological activity of the BP samples and be able to better compare the obtained results with other works (Marghitas, 2014). Three spectrophotometric procedures, namely DPPH, FRAP, and ABTS assays, were used to determine the antioxidant capacity of BP samples. The results are summarized in Table 7.

Table 11: DPPH and ABTS scavenging capacity and FRAP assays results

| Sample | DPPH $EC_{50}$ (mg/mL) | TEAC (mM trolox/mg extract) | FRAP (mg GAE/g extract) |
|---|---|---|---|
| P1 | 0.38 ± 0.00 | 0.98 ± 0.02 | 0.02 ± 0.00 |

| | | | |
|---|---|---|---|
| P2 | 0.15 ± 0.00 | 1.33 ± 0.02 | 0.03 ± 0.00 |
| P3 | 0.25 ± 0.01 | 1.22 ± 0.15 | 0.09 ± 0.01 |
| P4 | 0.37 ± 0.00 | 1.59 ± 0.15 | 0.02 ± 0.01 |
| P5 | 0.75 ± 0.04 | 1.11 ± 0.05 | 0.03 ± 0.00 |
| P6 | 0.20 ±0.02 | 1.35 ± 0.05 | 0.04 ± 0.01 |
| P7 | 0.07 ± 0.00 | 1.34 ±0.16 | 0.05 ± 0.02 |
| M1 | 0.71 ± 0.00 | 0.83 ± 0.13 | 0.03 ± 0.00 |
| M2 | 0.52 ± 0.01 | 0.90 ± 0.01 | 0.05 ± 0.01 |
| M3 | 0.41 ± 0.01 | 0.87 ± 0.03 | 0.03 ± 0.01 |
| M4 | 0.28 ± 0.01 | 0.97 ± 0.03 | 0.03 ± 0.01 |
| M5 | 0.45 ± 0.01 | 1.01 ± 0.07 | 0.04 ± 0.01 |
| M6 | 0.29 ± 0.01 | 1.10 ± 0.05 | 0.04 ± 0.01 |
| M7 | 0.28 ± 0.00 | 1.26 ± 0.02 | 0.04 ± 0.02 |
| M8 | 0.68 ± 0.05 | 0.81 ± 0.02 | 0.51 ± 0.02 |

4.4.1.1.DPPH scavenging activity

The DPPH scavenging assay is one of the few stable organic nitrogen free radicals and one of the most frequently used, and therefore, most suitable for comparison with other studies. Its capacity is attributed to the extract's hydrogen-donating potential (de Oliveira *et al.*, 2012), being its radicals sensitive to hydrogen donors' presence which allows the scavenging to operate at low concentrations (Mohdaly *et al.*, 2015). The extract concentration required to obtain a 50% of the antioxidant effect, the $EC_{50}$, was used for the expression of the results, meaning a lower $EC_{50}$ value a powerful antioxidant potential. The $EC_{50}$ of ethanol extracts of the 15 bee pollen samples ranged from 0.07 ± 0.00 to 0.75 ± 0.04 mg/mL, Table 7. *Castanea* bee pollen from Portugal showed the highest antiradical activity with an $EC_{50}$ of 0.07 ± 0.00 mg/mL. This value is in accordance with the results reported in the literature, where the best $EC_{50}$, with a value of 0.09 ± 0.06, was obtained for chestnut pollen from the black sea region of Turkey (Avşar et al., 2016). Following P7 best antiradical activity, P2 bee pollen, with a value of 0.15 ± 0.00 demonstrates that Oleaceae's bee pollen may present a good radical scavenging activity, which is in accordance with previously reported results(Campos *et al.*, 2003). Methanolic extract of three *Echium* bee pollen samples from Portugal showed an approximately $EC_{50}$ mean value of 3 mg/mL which is lower than the value of 0.75 ± 0.04, presented by P5 *Echium* sample in this study.

Among the Moroccan samples, M4 (*Olea europea)* presented the best $EC_{50}$ with a value of 0.28 ± 0.00 mg/mL. M7 and M8 belong to the same botanical family, Fabaceae, and contained the same pollen grains genera, however, they showed divergent values, M8 a value of 0.68 ± 0.05 mg/mL whereas M7 with o0.28 ± 0.00 mg/mL. This divergence can be explained by the different environmental conditions of both geographical origins, as M7 was collected from a mountains region, while M8 was gathered from beehives located in an oasis.

Previous investigations pointed out that antioxidant activity is related to the flavonoids and phenolic compounds content (Campos *et al.*, 2003; Feas *et al.*, 2012). In the current study, a positive correlation was observed between the DPPH and the TPC. However, DPPH and TFC indicated a poor correlation shown in table8. Kocot *et al.*, (2018) explained that some researchers already confirmed a strong positive correlation whereas others did not note a considerable relationship between the antioxidant capacity of bee pollen and its phenolic and flavonoid content.

4.4.1.2. Reducing power assay

The capability for reducing Fe(III) is another bioactivity evaluation method, working as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Avşar *et al.*, 2016). The results, expressed in gallic acid equivalents, are shown in Table 7. In the current study, the reducing power values varied from 0.10 ± 0.01 to 0.02 ± 0.01 mg GAE/ g. The highest reducing power value was registered for the P3 sample, with a value of 0.09 ± 0.01 mg GAE/ g, which also showed a high total flavonoid content compared to other samples in our study. Also, have shown similar results where samples, harvested from the northwestern of Transylvania, belonging to the Rosaceae family presented the highest reduction rates between other families like Fabaceae and Asteraceae. However, Marghitas, (2014) registered the highest reducing power for an Asteraceae *Matricaria* bee polen from Romania, which is the opposite of our findings, where the two Asteraceae BP samples of our study, P1 (Asteraceae *Carduus*) and M3 (Asteraceae *Taraxacum*), presented relatively a low reducing power of 0.02 ± 0.00 and 0.03 ± 0.00 mg GAE/ g respectively.

Table 12: Pearson's correlation coefficient results

| Pearson | TEAC (mM trolox/mg BP) | DPPH (EC50) | FRAP (GAE mg/g extract) |
|---|---|---|---|
| **TPC (GAE mg/g extract)** | -0.7 | -0.7 | 0.0 |
| **TFC (QE mg/g extract)** | -0.5 | -0.5 | 0.2 |

#### 4.4.1.3. ABTS Radical Scavenging Activity Assay

The ABTS method is considered, experimentally and instrumentally, simple to apply, provides reproducible data, and consequently is widely reported (Ilyasov *et al.*, 2020). Generally, the number of hydroxyl groups in phenolics correlates with the ABTS scavenging activity but some outliners were previously reported (Ilyasov *et al.*, 2020). Previously, Mohdaly *et al.*(2015) demonstrated that the ABTS radical cation is more reactive than the DPPH radical. In the present research, the highest antioxidant potential was registered for the P4 and P6, which represent the *Rubus* genus, with 1.59 ± 0.15 and 1.35 ± 0.05 mM Trolox/mg BP, while c*astanea* bee pollen showed acceptable scavenging capacity with 1.34 ±0.16 mM Trolox/mg BP. Generally, the results of ABTS scavenging activity from Portugal and Morocco were better than those reported previously in multi-floral bee pollen from Brazil, with a mean value of 0.12 mM trolox/g BP (Almeida et al., 2017). Moreover, a study on BP from Romania revealed that Salicaceae bee pollen extract possessed a highest TEAC value of 6.84 mM Trolox/g BP which is lower than the BP values registered in the current study (Marghitas, 2014).

### 4.4.2. Anti-tumor activity

Each bee pollen extract was screened for potential *in vitro* cytotoxicity activity against human cancer derived as well as a non-tumor cell lines at a single concentration of 20 mg/mL. The growth inhibition (GI) of the cells was not significant in most of the samples ($GI_{50}$>1000). Among the Portuguese samples, table 13, P1, P6 as well as P7 were the only samples that showed cytotoxicity effect exclusively against MCF-7 (breast adenocarcinoma).

In terms of Morrocan extracts, M1 and M4 displayed an anti-tumor capacity against MCF-7 and HeLa respectively, shown in table 14. The lowest $GI_{50}$ observed was

shown by P1 sample against MCF-7, with a value of 17 µg/mL. However, P6, P7 and M1 cytotoxicity effect varied between 734±7 and 814±10 µg/mL. Phenolic compounds were mentioned to exert anti-carcinogenic and anti-mutagenic activity in addition to the antioxidant one (Tohamy et al., 2014). Denisow and Denisow-Pietrzyk, (2016) added that anti-carcinogenic properties of BP may be originated from its antioxidant capacity such as the elimination of oxygen reactive species.

Therefore, it was reported by Kustiawan *et al.*, (2014) that certain phenolic compounds possessed anti-tumor activity against specific cell lines. For instance, kaempferol exhibited a particular cytotoxicity to ChaGo I (lung undifferentiated cancer) as well as KATO-III (gastric carcinoma) cell lines and naringenin to BT474 (ductal carcinoma). Similarly, Ravishankar *et al.*, (2013) mentioned the ability of quercetin in the down-regulation of oncogene expression as well as the up-regulation of tumor suppressor genes. Quercetin, kaempferol and other flavonoids, already detected in BP, were responsible for antiprostatic activity (M. Campos et al., 1997). In the current study, samples that showed an anti-tumor capacity against MCF-7, presented different phenolic compounds profiles with different concentrations, however, P1 and P7 contained majorly isorhamnetin glycosides. Isorhamnetin, in the aglycone form, presented a vast anti-carcinogenic effect that was proven against breast cancer cells as MCF-7, colon cancer cells as Caco-2 and cervical cancer as HeLa (Gong et al., 2020). Also, P6, contained a prominent amount of methyl herbacetin glycosides especially methyl herbacetin-O-dihexoside which was detected abundantly in a bee bread from the same geographical origin (Sobral et al., 2017) and both samples presented an anti-tumor capacity against MCF-7.

Table 13: Cytotoxicity activity ($GI_{50}$ values, µg/mL) of the Portuguese BP samples

| **Cell lines** | **$GI_{50}$** | | | | | | |
|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | P6 | P7 |
| **AGS** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **CaCo** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **HeLa** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **hFOB** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **MCF-7** | 17±1 | >1000 | >1000 | >1000 | >1000 | 814±10 | 746±1 |
| **NCI** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |

Concerning M1, the major flavonoid detected was kaempferol-3-*O*-rhamnoside which Lee, Choi and Hwang, (2017) confirmed the aglycone's metastasic-related behaviours against MCF-7.

Finally, M4 was the only sample that showed an anti-tumor potential against HeLa cells that can be explained by the important quercetin-diglucoside amount detected. Quercetin antiproliferative effect was already proven and explained by Kim *et al.*, (1984) and Sundaram *et al.*, (2019) as this flavonol induce apoptosis in HeLa cells through DNA fragmentation. Our GI results were lower compared to the ones reported previously by Falcão *et al.*, (2019) in Moroccan propolis, as they marked significant GI of tumor cells in all experiments while some samples showed an anti-tumor capacity on more than one cell line. In the same study, $GI_{50}$ of two different samples were around 10 µg/mL, against HeLa cells, which was a close value range to the standard's one (ellipticine).

Table 14: Cytotoxicity activity ($GI_{50}$ values, µg/mL) of the Moroccan BP samples

| **Cell lines** | **$GI_{50}$** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 |
| **AGS** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **CaCo** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **HeLa** | >1000 | >1000 | >1000 | 495±6 | >1000 | >1000 | >1000 | >1000 |
| **hFOB** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **MCF-7** | 734±7 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| **NCI** | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |

Kustiawan *et al.*, (2014) tested the cytotoxicity capacity of honey, bee pollen and propolis crude extracts harvested from stingless beehives in Indonesia against different cell lines. They found out that BP extracts were the least cytotoxic and confirmed that bee products' bioactivities rely on the bees species, harvesting method, geography, season as well as the extraction method.

Compared to chemotherapy's drugs, the observed carcinogenic effect of BP extracts, rich in flavonoid, still considered low, however, Kustiawan *et al.*, (2014) suggested chemical modification of the mentioned flavonoids in order to improve their biological capacity.

## 5. Conclusion

In this study, the floral origin of 15 bee pollen samples was determined with palynological analysis. The TPC, TFC and the antioxidant capacity of the Portuguese samples showed higher values than the Moroccan ones. However, our findings did not show any correlation. The LC-ESI-MS analysis results revealed the identification of 98 phenolic compound, 76 in the Portuguese samples and 50 in the Morrocan ones. The identified compounds were present in the following order, flavonoid glycosides > phenylamides > phenolic acids. Only one flavonone, the naringenin, was detected in P2, Oleaceae *Ligustrum* bee pollen, however phenolic acids were not detected in the none of Portuguese samples. The GC-MS analyses resulted in the identification and quantification of 81 volatile compounds, which 50 VOCs were detected in the Portuguese samples and 49 in the Moroccan ones.

The results confirmed that bee pollen from the same floral origin tend to have the same phenolic compounds profile which is the case of P4 and P6, Rosaceae *Rubus* bee pollen, which were collected from different regions of Portugal. Also, those samples presented a lot of similarities in the volatile compounds profile. Based on that, both profiles can be used to discriminate floral origins of bee pollen without fulfilling palynological analysis. In addition, some volatile compounds can be used as biomarkers even for geographical origin discrimmination. Chestnut bee pollen from Portugal showed high DPPH and ABTS scavenging activity as well as a relatively important reducing power. The same sample presented particularly a rich phenolic profile, with a rich composition around 90% in phenylamines, which are the main responsible of the important antioxidant activity.

Concerning the anti-carcinogenic effect, bee pollen extracts did not demonstrate generally a strong capacity however, only P1, Asteraceae *carduus* bee pollen, exhibited an anti-tumor potential against MCF-7 (breast carcinoma).

## 6. Future perspectives

- The phenolic extraction needs to be optimized as the ethyl acetate fraction of BP's ethanolic extraction may offer better recovery of PCs while analyzing the TPC.
- Some phenolic compounds were detected but not identified which needs nuclear magnetic resonance analysis to fully recognize the molecules structural characteristics.
- Phenolic compounds may show better bioactivity results, as the antioxidant one, if tested for its anti-inflammatory, anti-bacterial or hepatoprotective activities
- For a better anti-tumor screening, individual compounds can be isolated and tested for their bioactivity against cell lines which will help emphasizing specific benefits of particular phenolic compounds.
- Volatiles and semi-volatiles contained in our samples may be tested for their biological activity.
- Principal Component Analysis (PCA) can describe better the bee pollen's composition and as a result provide better floral origins discrimination results.

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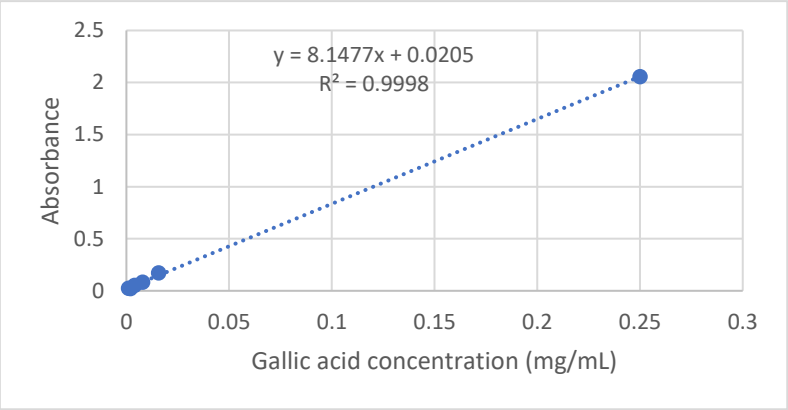
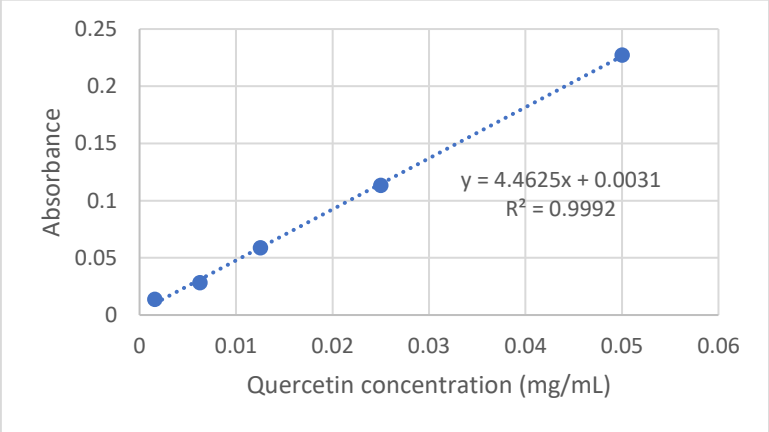
# Appendix

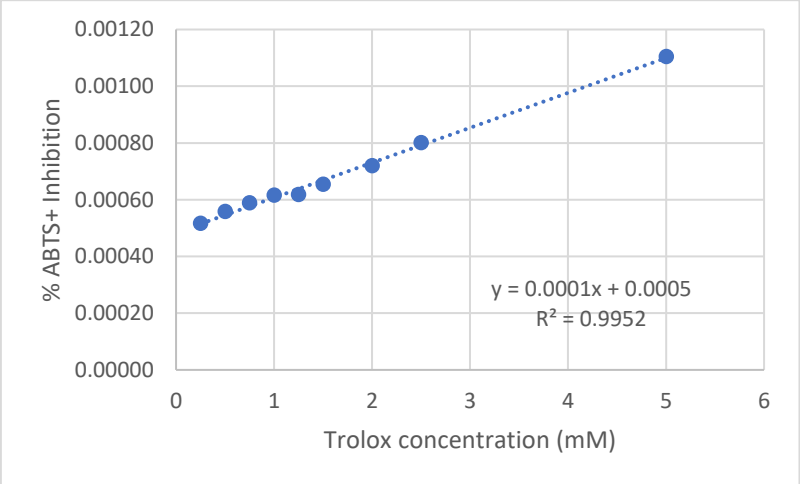
Calibration curve of the TPC assay



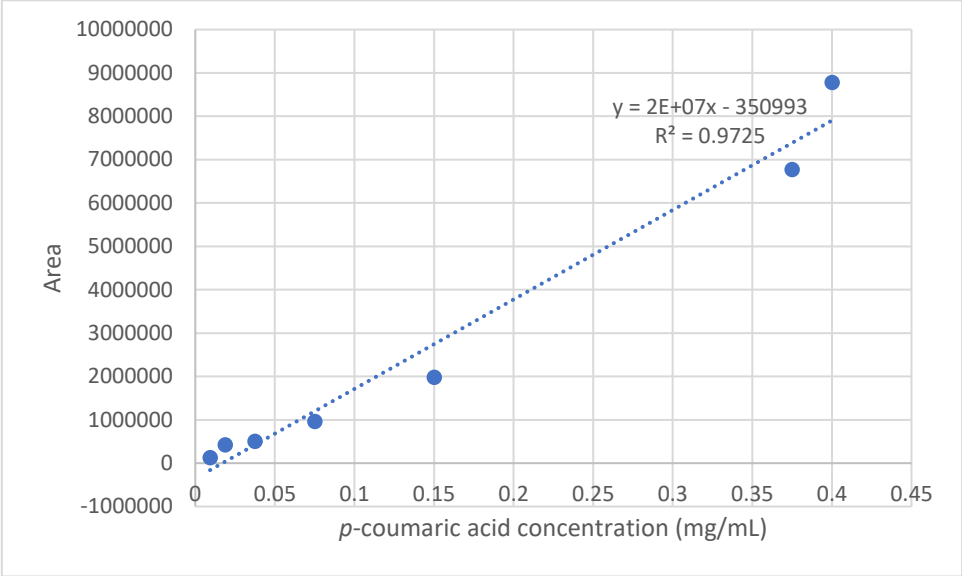

Calibration curve of the TFC assay

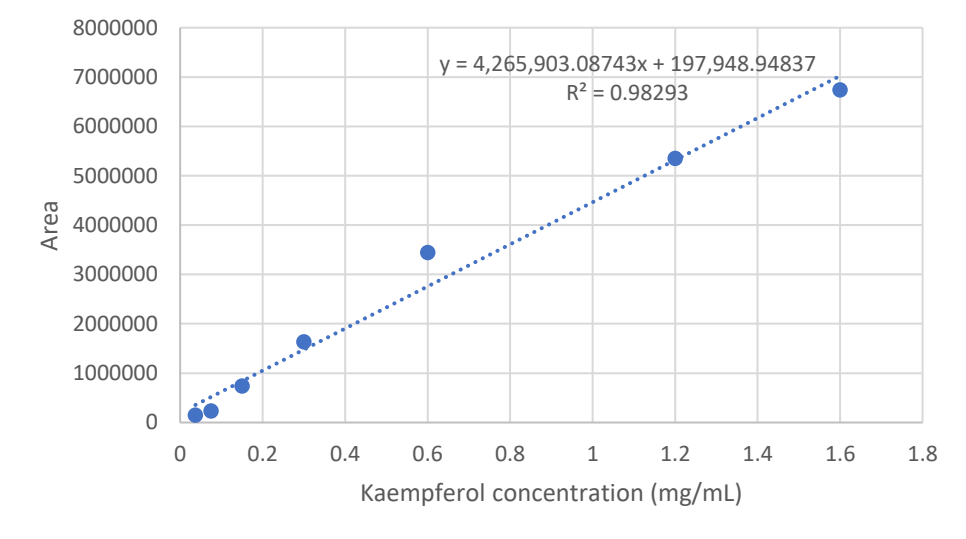


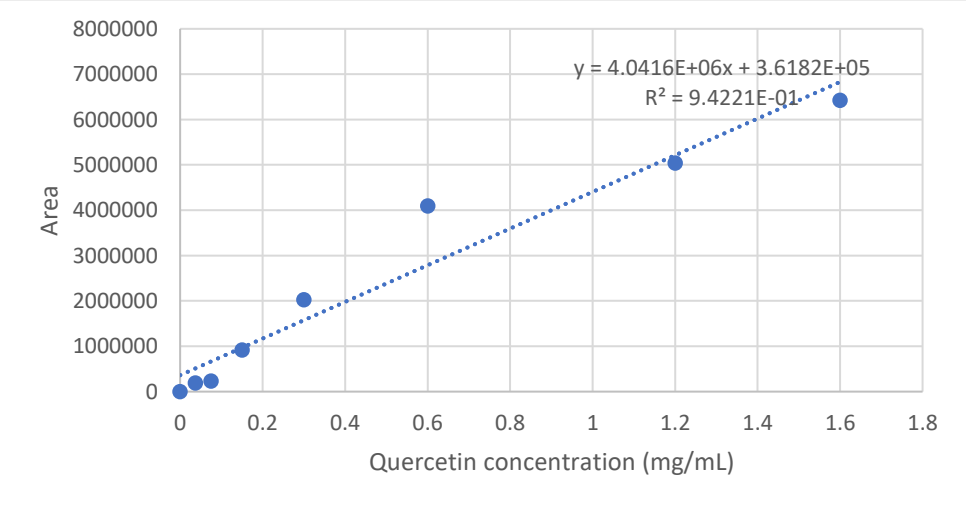

Calibration curve of the ABTS scavenging assay

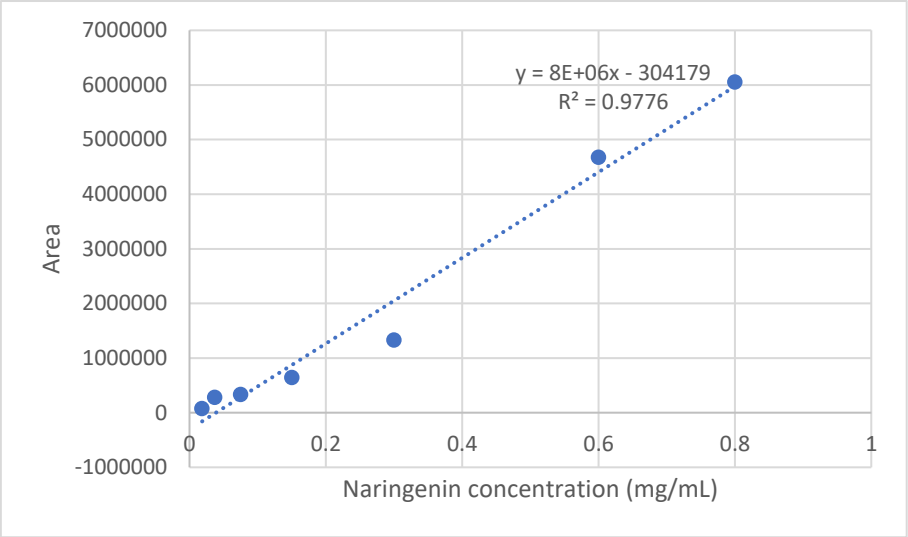


HPLC quantification calibration curves

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Naringenin concentration (mg/mL)
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1