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DIMITRA KAROUSOU
Graduate Agronomist

"Enrichment of Samothrace honey with bioactive components of the *Arbutus andrachne* plant"

Supervisor: Gerasopoulos Dimitrios, Professor

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Dimitra Karousou

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Postgraduate Dissertation

<u>Examination board</u>

Gerasopoulos Dimitrios, Professor Mourtzinos

Ioannis, Assistant Professor Koutsoumanis

Konstantinos, Professor

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"Enrichment of Samothrace honey with bioactive components of the *Arbutus andrachne* plant"

Abstract

Honey is a natural product derived either from the secretions of insects or from the nectar of blossoms, which bees collect, transform and store until maturity. Glucose, fructose, water, and amounts of protein, minerals, organic acids, and vitamins are its key components. In the present dissertation it was studied the honey of Samothrace (Samothraki), a type of honey of particular interest for which there are no bibliographic references. Along with honey, various extracts (blossoms, leaves, fruits) of the *Arbutus andrachne* plant were studied, some of which were then added to honey for the purpose of producing an enriched type of honey. Three different solvents, water, ethanol-water solutions and β-cyclodextrins were used for the extraction of the components. Total phenolics, antioxidant capacity, total flavonoids and arbutin content were determined using HPLC. The antibacterial activity of Samothrace honeys was further determined using the Agar Well Diffusion Assay against three food pathogens: *Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes*.

Total phenolics in Samothrace honeys averaged 590mg GAE/L, antioxidant capacity at about 2.33mMTRE and total flavonoids around 1.12mMQE. Manuka MGO 30+ honey was used as a positive control, which was well above the rest of the honey, with the exception of total flavonoids. Total phenolics were 2533mgGAE/L of over-ripe leaf extract with solvent 80 ethanol: 20 water, while with β-cyclodextrin it was 7828mgGAE/L. The antibacterial activity of Samothrace honey was greater than that of the Manuka honey control in most cases, with *Staphylococcus aureus* being the most susceptible microorganism. Enriched honeys, at the highest enrichment rate (5%), reached a total phenolic concentration of 1390mgGAE/L.

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Chapter 1– Introduction

In recent years there has been a trend towards healthier, more nutrient-dense foods with no chemical additives and a positive impact on human health. Both consumers and the food industry are gradually turning to functional foods, enriched with natural ingredients, little to no processed.

Honey is a special product, with a very characteristic taste and aroma, which is not particularly processed in order to maintain its quality characteristics and properties. In an overall picture it is a food product that is considered completely natural and quite nutritious.

The specific Samothrace honey presented in this dissertation is a type of honey of particular interest, which has not been studied before. It is a type of honey that is characterized as blossom honey. On the island of Samothrace, 1441 species of endemic plants have been found and recorded, belonging to 559 genera and 107 families (Biel & Tan, 2014), which testifies to the enormous biodiversity of the island. That is why the various plant species, many of them endemic, as well as the predominance of two plants, *Arbutus andrachne* and *Erica arborea*, which participate in honey, transfuse special characteristics to it.

The *Arbutus andrachne* plant, which could be attributed as wild arbutus, is a plant related to the known arbutus (*Arbutus unedo*). Arbutus, both as a plant and arbutus honey, which results from the autumn blossoms of the tree, has been studied several times by the global research community, and it is even known for its properties. However, while the *Arbutus andrachne* plant has been studied to some extent for its properties, the spring honey of Samothrace, which contains percentages of this plant, has not been evaluated for its chemical characteristics.

The purpose of the dissertation is initially to determine the chemical characteristics (total phenolics, antioxidant capacity, flavonoids) both of the parts of the *Arbutus andrachne* plant (blossoms, leaves, fruits) and of Samothrace honey. For Samothrace honey, Manuka MGO 30+ is used as a positive control, known for its antioxidant and antibacterial properties from many publications. In this context, the antibacterial activity of Samothrace honey against three food pathogens is also tested: *Staphylococcus aureus*, *Escherichia coli* and *Listeria*

monocytogenes.

In addition, the arbutin content both in the various parts of the *Arbutus andrachne* plant and in Samothrace honeys is determined using High Performance Liquid Chromatography.

Finally, the ultimate goal of the experiment is to achieve, after various tests, the successful enrichment of Samothrace honey with plant extracts from the *Arbutus andrachne* plant in various percentages and to determine again the chemical characteristics, the arbutin content as well as the antibacterial activity of this new enriched honey.

Chapter 2 - Literature review

2.1 Honey

Honey is a natural biological product derived from flower nectar and honey secretions, which bees collect, and after many years of research it has been proven to have advantages for humans both as a medicine and as a food. Glucose, fructose, water, and amounts of protein, minerals, organic acids, and vitamins are its key components. Consumers prefer it for its distinctive taste, sweetness and texture. In the process of extracting honey from honeycombs it contains small amounts of pollen, beeswax and other undesirable materials, which are removed by a filtering process and ultimately clarification time for better product quality, consumer acceptance and increased shelf life (Subramanian et al., 2007).

Honey is the only nutritionally acceptable form of concentrated sugars worldwide (Ferreira et al., 2009; Meda et al., 2005). In addition to food, it is also used as a food preservative. In recent years, particular importance has been given to the functionality of foods that have honey as their preservative (Ferreira et al., 2009).

According to Community Legislation (2001/110/EC), Honey is the natural sweet substance produced by *Apis mellifera* bees 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.

The honey produced is divided into two major categories. The first includes blossom honeys, widely known, and at the same time most often consumed, produced from nectar (Figures 2.1.1-2). and flower sap, with the best known being the honeys from thyme, orange, cotton, heather, lavender, acacia, etc. The second category includes honey from honeydew (Figures 2.1.1-2), produced from excreta of the plants themselves or excreta of insects that suck the plants, such as the insect *Marchalina hellenica* which creates the cotton wool of the pine tree, from which the well-known pine honey comes. The insect absorbs the vegetable sap and in its digestive system, it is mixed with digestive juices rich in enzymes. The excess quantity that cannot be taken up by the insect is excreted by excretory organs in the form of small drops. These drops constitute the honeydew and they are collected by the bees which turn it into honey. The honeydew is richer in nutrients than the original vegetable sap, since passing through the insect it is enriched with various vitamins and other substances. This category also includes honey from fir, oak and other coniferous and forest plants.

Figures 2.1.1-2: Collection of nectar for the production of blossom honey (left) and collection of honeydew (right).

2.1.1 Components of honey

The composition of honey is relatively variable and depends primarily on its plant origin. At the same time, certain external factors, such as environmental factors or the microclimate of the apiary area and the processing to which it is to be subjected, contribute considerably. Honey is a solution of sugars, of which fructose (38%) and glucose (31%) are the predominant sugars, along with phenolic compounds, minerals, proteins, free amino acids, enzymes and vitamins as secondary components (Alvarez-Suarez et al., 2010; Spilioti et al., 2014).

Although honey has been found to contain about 200 types of different substances(Cianciosi et al., 2018; Ferreira et al., 2009; Spilioti et al., 2014), it is generally about 80% sugars. Sugars are organic compounds of small to large molecular weight, providing the necessary energy that living organisms need for vital functions, and at the same time they are structural components of cell membranes and cells in general, both in animal and plant organisms. In analyses on honey, at least 22 different sugars have been found. The average sugar composition in a type of honey (Table 2.1.1.1.) differs depending on whether it is blossom honey, or honey from honeydew (Thrasyvoulou & Manikis, 1995). The sugars that we find in very high concentrations in honey(Figure 2.1.1.1) are mainly the monosaccharides fructose and glucose, sugars that are not present in nectar or honeydew of plants, but come from the hydrolysis of sucrose in the bee crop. During the honey production process, bees secrete the enzyme invertase from their hypopharyngeal glands, which breaks down sucrose into glucose and fructose (Crane, 1990).

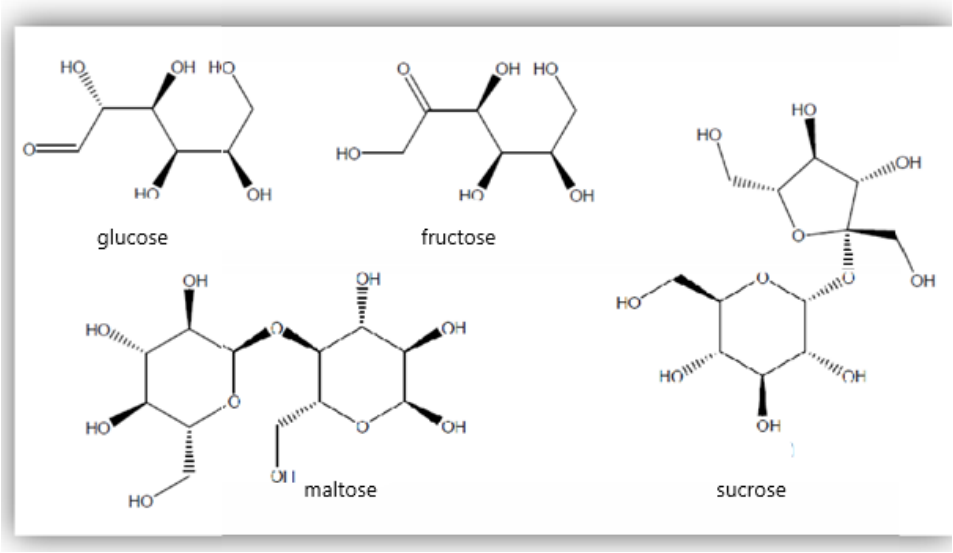


Figure 2.1.1.1: Chemical structures of the most important sugars in honey

Table 2.1.1.1.: The most important components of honey on average values (Cianciosi et al., 2018)

Components	Quantity per 100g of honey
Water	**16-18g**
Carbohydrates (Total)	**65-73g**
Fructose	**35-41g**
Glucose	**25-28g**
Maltose	**1-2g**
Sucrose	**0.2-1.2g**
Proteins, vitamins, amino acids, minerals	**0.5-3g**

Apart from the large percentage of sugars, honey contains many substances, such as organic acids, amino acids, enzymes, polyphenols, minerals, vitamins in smaller percentages.

The most important organic acid of the ones contained in honey is gluconic acid, which is produced in honey by the effect of the enzyme glucose oxidase(Figure 2.1.1.2), a reaction in which hydrogen peroxide is formed(Cianciosi et al., 2018). Hydrogen peroxide and other compounds, such as some carboxylic acids, are responsible for much of the antibacterial activity of honey, since they are responsible to some extent for the reduced pH value of honey (Cianciosi et al., 2018). Other organic acids found in honey are: aspartic, citric, acetic, formic, fumaric and galacturonic.

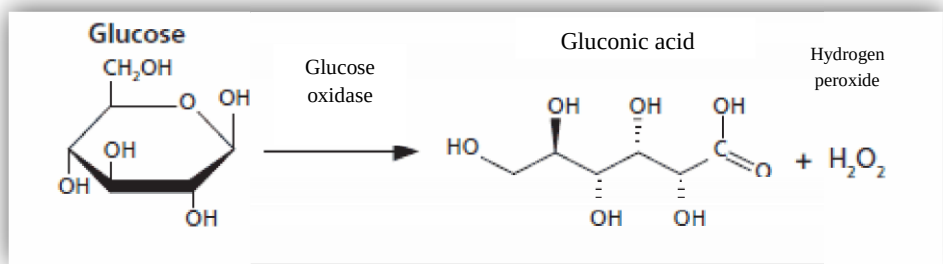


Figure 2.1.1.2: Effect of the enzyme glucose oxidase (Cianciosi et al., 2018)

The most important of the amino acids is proline (Figure 2.1.1.3), which is also more abundant. Proline holds 50-85% of the total amino acids of honey. It is secreted by the hypopharyngeal glands of bees and aims to regulate the addition of invertase to nectar (Charizanis, 1996). It is also used as an assessing parameter for honey maturation (Meda et al., 2005). Other amino acids found in honeys are: alanine, phenylalanine, tyrosine, glutamic acid, isoleucine and leucine.

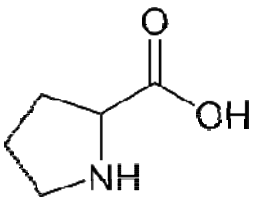


Figure **2.1.1.3:** Chemical structure of the amino acid proline

The main enzymes contained in honey are invertase, glucoxidase or glucose oxidase and diastase, derived from the hypopharyngeal glands of bees, and catalase and acetic phosphatase, which are of plant origin (Crane, 1990). More specifically, invertase breaks down sucrose into glucose and fructose, glucose oxidase oxidizes glucose to gluconic acid and hydrogen peroxide in the presence of water, diastase or amylase breaks down starch and, although its role is not clear, it may help bees digest pollen. Catalase regulates the action of glycoxidase by controlling the equilibrium of hydrogen peroxide, and acetic phosphatase has no clear role, but it has been found in pollen, nectar, and honey.

Regarding polyphenols, honey contains significant amounts of: phenolic acids, flavonols, flavones, flavanols, flavonones, anthocyanidins, isoflavones (Figure 2.1.1.4). All of these compounds are often the product of secondary plant metabolism and are characterized by the presence of multiple phenolic groups exhibiting more or less complex structures. Phenolic acids carry a phenolic ring and at least one branch of organic carboxylic acid. The phenolic composition of honey depends mainly on its plant origin and can even be used as a tool for classification and authentication, especially in the case of monophytic honeys. The most common phenolic acids are gallic acid, coumaric acid, caffeic acid and benzoic acid.

Flavonoids carry two benzene rings joined by a linear chain with three carbon atoms, they usually have at least two hydroxyl groups and are often linked to sugars, constituting glycosides. Honey flavonoids may come from propolis, pollen, but also nectar or honeydew that bees collect from plant or insect secretions (Tomas-Barberan et al., 2001). The most common flavonoids are pinokebrin, pinobacnsin, chrysin, quercetin, and methyl esters of kaempferol.

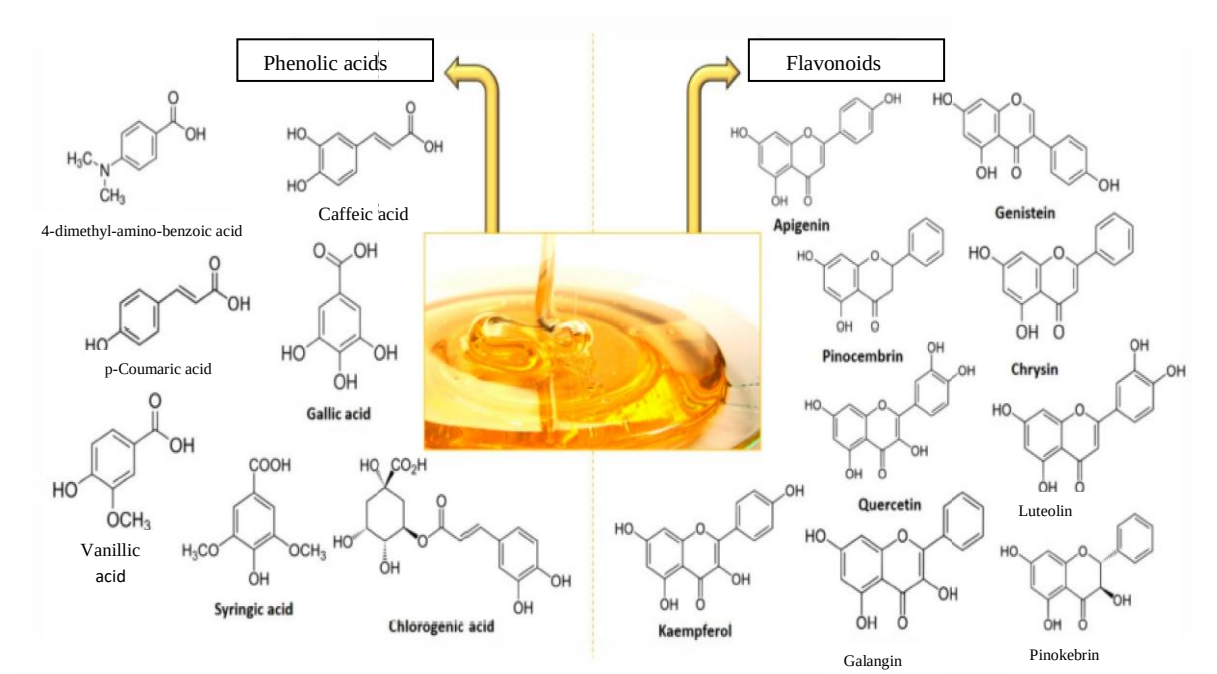


Figure 2.1.1.4 : Honey polyphenols: phenolic acids and flavonoids (Cianciosi et al., 2018)

Polyphenols, in terms of their type and concentration, depend on the plant origin and are the main substances responsible for the antioxidant properties of honey (Figure 2.1.1.4), stabilizing free radicals by freeing a hydrogen from their hydroxyl group and the degree of their activity is related to the number of hydroxyl groups they carry in their molecule (Cianciosi et al., 2018).

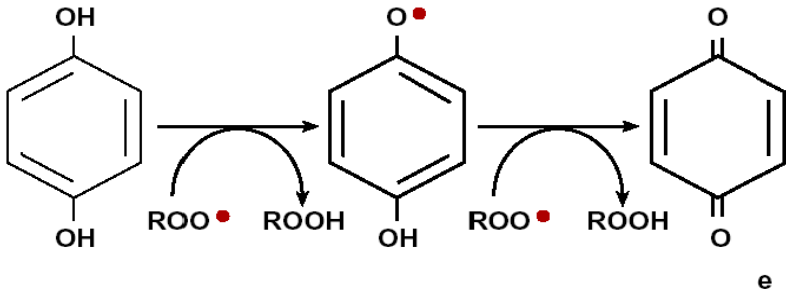


Figure 2.1.1.4 : Mechanism of antioxidant activity of phenolics (Sandhya et al., 2010)

Other important components of honey, which appear in smaller quantities of course, are minerals (K, Ca, Cu, Fe, Mg, Mn, F, Na, Zn, Se) and vitamins (C, B1, B2, B3, B5, B6) with the latter coming almost exclusively from pollen.

2.1.2 Properties of honey

Not at all negligible is the number of studies showing that honey has a variety of functional properties in humans, which help promote health and depend largely on the source of the flora from which each honey comes. These properties could be associated with the high osmotic capacity of honey, due to high sugar concentration, antimicrobial properties (Willix et al., 1992) and antioxidant capacity (Alvarez- Suarez et al., 2010).

The composition of the active components in plants depends on many different factors. Some of them are biological, plant and climatic, which is why the properties of honey from different locations, with different climate, possibly from different years of production in the same area, are different (Alvarez-Suarez et al., 2010).

Honey has been used to treat burns, gastrointestinal disorders, asthma, infected and chronic wounds, skin ulcers, cataracts and other eye diseases. This beneficial role is partly attributed to antibacterial capacity (Ferreira et al., 2009). But since some of these diseases are a consequence of oxidative damage, it seems that some of the healing properties of honey are due to its antioxidant capacity. In addition, the presence of hydrogen peroxide as well as metals such as copper and iron can lead to the formation of highly reactive hydroxyl radicals as part of the antibacterial system (Alvarez-Suarez et al., 2010).

2.1.3 Antioxidant activity

A free radical is an atom, a molecule, or a compound that is highly unstable due to its atomic or molecular structure. Free radicals, whatever their origin, are very active since they try to create bonds with other molecules, atoms, or even individual electrons in order to manage to create a chemically stable compound. Therefore, oxygen radicals (ROS) and free radicals cause molecular transformations and gene mutations in many types of organisms. This is called oxidative stress and it is known to either be the starting point or cause of many different diseases (Gul & Pehlivan, 2018).

An antioxidant is a molecule that has the ability to slow down or prevent the oxidation of other molecules, managing to neutralize the loads of free radicals, weakening them. In recent years, many scientific studies have shown that substances with antioxidant activity reduce the risk of chronic diseases, such as cancer and heart disease. Important sources of natural antioxidants are cereal grains, fruit and vegetables. Many components have been recognized as powerful antioxidants, such as: vitamins C (ascorbic acid) and E (tocopherol),

carotenoids, phenolic acids. In terms of chemical structure, these substances belong to different groups, and therefore exhibit a variety of physicochemical properties.

Natural and artificial antioxidants have been used for many years to preserve food, helping to delay spoilage, reduce rancidity or discolouration due to oxidation caused by light, heat and some metals. Antioxidants owe their antioxidant capacity to both enzymatic substances, such as catalase and glucose oxidase, and non-enzymatic ones, such as organic acids, amino acids, Maillard reactions, proteins, flavonoids, phenolic acids (Alvarez-Suarez et al., 2010, Meda et al., 2005), tocopherols, flavonols, catechins, ascorbic acid and carotenoids (Ferreira et al., 2009).

Honey, in particular, contains many substances with antioxidant activity, such as flavonoids and other phenolic components, peptides, enzymes, organic acids, minerals and trace elements, products of the Maillard reaction and other components (Gheldof et al., 2002). The antioxidant activity of each honey varies and depends on its individual components. In a research that evaluated the colour of honeys and correlated it with antioxidant activity (Ferreira et al., 2009), dark honeys were shown to have stronger antioxidant activity, since they were richer in phenolic substances and minerals. It is very important to stress that it was found that these honey substances are readily bioavailable to the human body (Schramm et al., 2003). Honey flavonoids contribute significantly to its antioxidant activity. Some of those derived from propolis, such as pinocebrin, pinobanksin and chrysin, often make up the bulk of the total flavonoids in honey, while others are of plant origin.

Moreover, honey, as a source of antioxidants, has been shown to be effective against the oxidation reactions caused by light, heat and some minerals that make food unacceptable by the consumer as well as non- suitable for consumption such as: the enzymatic browning of fruit and vegetables, the oxidation of lipids in meat and the inhibition of the growth of food pathogens (Ferreira et al., 2009).

2.1.4 Antibacterial properties

The inhibition activity of honey against microorganisms has been attributed to several key properties of honey, including the osmotic effect, the low pH, the hydrogen peroxide production (Weston et al., 1999; Willix et al., 1992), as well as the presence of phenolic acids, lysozyme, and flavonoids (Patton et al., 2006; Snowdon & Cliver, 1996).

Honey has the ability to inhibit or kill most microorganisms, due to: a. the high sugar and low water content that create an osmotic pressure that kills microorganisms, the relatively

low pH (3-5), b. the glucoxidase system that produces H_2O_2, a substance toxic to microorganisms, c. the low protein content that is not attractive to microorganisms, d. the O_2 that cannot penetrate honey due to the high viscosity, so aerobic microorganisms cannot survive in the honey system. In addition, some specific substances in honey such as: pinocebrin, lysozyme, phenolic acids, terpenes, benzyl alcohol and various volatile components are lethal to microorganisms.

Honey has been used as a wound healer since ancient times. Many reports mention an inhibition activity of honey against many bacteria of particular clinical importance. The healing of epidermal wounds is related to the physical property of osmosis and the antibacterial properties mainly of hydrogen peroxide (Taormina et al., 2001). Hydrogen peroxide is produced by the enzyme glucose oxidase which is found in the hypopharyngeal glands of bees. In honey there is also catalase which comes from the pollen of the blossoms. The higher the glucose oxidase levels or the lower the catalase levels are, the higher the peroxide levels are too (Taormina et al., 2001). However, there are also non-peroxide agents that affect the antimicrobial properties of honey. Such are the lysozyme, the phenolic acids and the flavonoids present in honey. Flavonoids are mostly derived from propolis, a resinous secretion collected by bees and used as antimicrobial protection of hives. Phenolic compounds are also found in nectar, have particular antioxidant properties and have been studied for their ability to inhibit the growth of gram-positive and gram-negative bacteria. These factors give honey very special, unique properties as a wound dressing. In particular, the inhibition of infections, the rapid suppression of wounds with inflammation, the minimization of scars and the stimulation of angiogenesis as well as the development of epithelium are ensured (Taormina et al., 2001). Of particular interest is the treatment of various food pathogens with natural antimicrobials in recent years.

In many researches in the world literature, the antibacterial activity of honey has been proven. These researches differ from each other in the origin of honey and the pathogen on which the interest is focused, but at the same time converge in the general antimicrobial behaviour of honey. Taormina et al. (2001) tested the behaviours of various honeys in cultures of various microorganisms, namely Escherichia coli, Salmonella typhimurium, Shigella sonnei, Listeria monocytogenes, Staphylococcus aureus and Bacillus cereus. They observed that at 25% concentration of honey, in the presence of catalase, inhibition was reduced, which implies that the antibacterial activity is strongly related to the presence of hydrogen peroxide. Darker honeys were not so affected by catalase, because they were found

have higher concentrations of antioxidants, which seem to contribute significantly to their antibacterial activity. They also observed that *Bacillus cereus* was the least affected of all microorganisms in the presence of honey, while *Staphylococcus aureus* appeared to be the most sensitive microorganism with the largest zones of inhibition observed in avocado and blueberry honeys. Later, Voidarou et al. (2011) studied coniferous, thyme, citrus and blossom honeys in cultures of *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Salmonella enterica* and *Streptococcus pyogenes*. They observed that coniferous and thyme honey gave the largest inhibitions of microorganisms, and that *Staphylococcus aureus* and *Escherichia coli* showed the largest inhibitions regardless of the origin of the honey.

Huttunen et al. (2012) studied five different honeys from plants: *Fagopyrum esculentum, Rubus chamaemorus, Calluna vulgaris, Vaccinium vitis-idaea* and *Epilobium angustifolium* and their activity against three microorganisms *Streptococcus pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus*. They observed that *Streptococcus pneumoniae* was susceptible to all honeys and *Streptococcus pyogenes* had significant inhibition in all honeys except honey from *Rubus chamaemorus*, which had no inhibition. *Staphylococcus aureus* showed strong inhibition by the honeys of *Fagopyrum esculentum* and *Calluna vulgaris*, while it showed no significant inhibition to no inhibition at all by the rest of the honeys.

The known antibacterial activity of Manuka honey was also of great interest to many researchers. In some researches, such as Deng et al. (2018), Anthimidou & Mossialos (2012) and Stagos et al. (2018), the buckwheat honeys, from Greece, Olympus, and Cyprus, which were compared to Manuka UMF +25 honey in terms of antibacterial activity, seem to have similar inhibitions to *Staphylococcus aureus* and *Pseudomonas aeruginosa* or even surpass it in some cases. In all three similar studies, *Staphylococcus aureus* appears to be again the most sensitive microorganism of the two. Nevertheless, in the study of Al-Nahari et al. (2015) Manuka UMF +20 honey seems to have the best inhibitions in *Pseudomonas aeruginosa* compared to two other honeys included in the comparison. This may be due to the non-peroxide antibacterial activity of Manuka honey, according to Snow & Manley-Harris (2004), since in their experiment Manuka honey did not give smaller zones of inhibition in the presence of catalase in the microorganism *Staphylococcus aureus*.

2.1.5 Qualitative characteristics

The quality of honey is mainly determined by its aesthetic, chemical, physical and microbiological characteristics. The most important physicochemical quality criteria of honey are defined by the Council Directive 2001/110/EC (EU, 2001) of the EU. The main criteria are moisture content, electrical conductivity, ash content, reducing and non-reducing sugars, free acidity, dissociation activity, and hydroxymethylfurfural (HMF) content.

2.1.6 Manuka honey

Manuka honey is a monofloral honey, derived from the plant *Leptospermum scoparium,* an endemic plant of New Zealand. The plant *Leptospermum scoparium* (Figure 2.1.6.1) belongs to the family of Myrtaceae and it grows as a shrub or small tree across New Zealand and eastern Australia. In traditional medicine, various parts of the plant are used as sedatives, to treat wounds, and against diarrhea due to their astringent properties. Manuka honey has been used for many years to clear infections including abscesses, surgical wounds, wounds, burns, and ulcers of varying etiology. Its efficacy in the form of wound dressing appears to be due to its non-peroxide antimicrobial properties. (Oelschlaegel et al., 2018).

Figure 2.1.6.1: Blossoms of *Leptospermum scoparium* (left) and whole plant (right).

It is therefore promoted, for this very reason, for its non-peroxide antibacterial activity (Mavric et al., 2008), which makes it highly sought-after and very expensive worldwide. Approximately 6000-8000 tons of Manuka honey are produced each year in New Zealand and they are available for export (Rogers et al., 2014). In addition to the antimicrobial activity of honey in general that exists due to the presence of hydrogen peroxide, Manuka honey has additional antibacterial activity, due to various phytochemicals derived from flower nectar.

Dihydroxyacetone(DHA), which is found in nectar, is the direct precursor for methylglyoxal (MGO) in honey, which is the most important bactericide that contributes to the additional bioactive properties of Manuka honey and based on this concentration, it is also advertised and promoted commercially (Burns et al., 2018).

Manuka honey is sought after because of its highly elevated antibacterial activity, which is marketed using a "Unique Manuka Factor" (UMF). UMF corresponds to the concentration (% w/v) of an aqueous phenol solution exhibiting the same antibacterial activity in a microbiological test of well diffusion against *Staphylococcus aureus* (Wallace et al., 2010). In addition to the UMF index, the MGO index, which shows the concentration of methylglyoxal in mg/kg, is the most common for the categorization of honey. Manuka honeys can start from MGO 15+, i.e. 15 mg/kg of methylglyoxal and reach up to MGO 550+, i.e. 550 mg/kg of methylglyoxal. Obviously, the price is also formed accordingly, surpassing $200/kg at the highest concentrations.

2.2 The *Arbutus andrachne* plant

The Ericaceae family has two evergreen species of *Arbutus* L. whose fruits are edible. *Arbutus andrachne* L. or "Greek strawberry tree", as it is often found in literature, appears in the wider Mediterranean region and southwest Asia. Its trees are relatively low in height usually less than 4m and their wood is used to make small furniture (Davis, 1982). *Arbutus unedo* L. is similar to *Arbutus andrachne* , but one of their main differences is that they differ in their flowering time, since the first one blooms in autumn and the second one in spring, in the number of lamina and in the positions of the branches (Serce et al., 2010).

The fruits of *A. andrachne* and *A. unedo* are also quite similar in shape and texture, since both are small fruit with a reddish colour externally (Figure 2.2.3-4). However, *A.unedo* has larger fruits, more flesh and is slightly more intense orange in colour. Natural hybrids of these two species, *A. andrachnoides*, have also been reported at various places in the East. These two species, *A. andrachne* and *A.unedo* , are not cultivated, but their fruits are collected and consumed either as fresh fruits or used for the preparation of jam, jelly, wine, spirits and liqueurs (Serce et al., 2010).

A special feature of the plant is the soft bark (Figure 2.2.1), which exfoliates in the summer, leaving a layer with a light green colour, which changes to a beautiful orange-brown one gradually. It blooms in spring and its blossoms are white or yellow-green (Figure 2.2.2). The fruits of the tree ripen in autumn (Abidi et al., 2015).

Figure 2.2.1-2: Characteristic peeling of *A. andrachne* bark (left), leaves and blossoms (right).

Figure 2.2.3-4: *A. andrachne* fruit (left) and *A.unedo* fruit (right)

2.2.1 Properties of the *A. andrachne* plant

Arbutus andrachne is found, as Atrooz et al. (2007) and Alzoubi et al. (2018) report, in many areas of Jordan, known as "Kaikab", and produces wild edible fruit. It is considered by some researchers and columnists to be an endangered species, as for example in the area of Palestine according to Aljabari et al. (2014). It is described as having clinical uses in arthritis, eczema, gout, rheumatism, and urinary tract disorders. It is also classified as a plant that affects the vascular system due to its astringent and uretic antiseptic properties by the United States Food and Drug Administration.

The plant showed strong antioxidant activity when 150 plant species from Jordan were tested for their antioxidant activities and overall phenolic content. The antioxidant activity of *A. andrachne* is mainly attributed to its phenolic components. Although its chemical profile is still largely untapped, it is reported in the research of Alzoubi et al. (2018) that it contains organic acids, phenolic and polyphenolic compounds (e.g. tannins and flavonoids), triterpenoids and sterols, phenolic and triterpenic acids, glycosides, vitamins and minerals. The antioxidant activity of these compounds is due to free radical removal activities. In their research, an experimental procedure is performed with the assumption that the methanolic extract of the fruits of *A.andrachne* will prevent chronic sleep disorders, which caused impairment of the hippocampus and memory, through its antioxidant properties.

There is generally a surge of interest in phytochemicals as new sources of natural antioxidants. The current goal and at the same time need of the market and of the consumer is to use natural antioxidants in food and pharmaceutical preparations in order to replace synthetic antioxidants.

Biological systems possess antioxidant enzymatic systems and chemical binders: a. endogenous enzymes (superoxide dismutase, glutathione peroxidase, catalase), which are all capable of removing oxygen from free radicals formed in cells and are thus protected from oxidative damage b. antioxidants obtained through diet (α-tocopherol, β-carotene, ascorbic acid, glutathione, uric acid), and c. some hormones (estrogens, angiotensin) (Tawaha et al., 2007).

Tissue damage resulting from the imbalance between oxygen free radical generation and removal systems (oxidative stress) has been implicated in the pathology of many disorders, such as atherosclerosis, cancer, diabetes, inflammatory joint disease, asthma, cardiovascular disease, immune system decline, and it could also play a role in aging processes (Tawaha et al., 2007).

Phenolic compounds generally exhibit different biological properties: antibacterial, anti-inflammatory, antiviral, anti-allergic, as estrogenic and immunostimulating agents.

It is also a very common plant in Turkey and it is found in many areas of Turkey. Leaf extracts are used as an antiseptic and diuretic substance, especially in traditional Western and Southern medicine. The plant

is also used as an antidiabetic agent. Various extracts obtained from the leaves of *A.andrachne* seemed to exhibit antimicrobial activity. In previous studies by Nahrstedt et al. (1992) for *A.andrachne*, monoterpenes, catechin, and epicatechin have been isolated. There are also other products related to triterpenoids, sterols, and lipids that are isolated from the bark, leaves, and fruit of *A.andrachne*.

Another study by Kamalak et al. (2010) studied and compared the nutritive value of young, old and senescent leaves of the *Arbutus andrachne* tree aiming at its use as part of herbivorous animal nutrition.

Tree foliage is an important component of the diet for ruminant animals such as goats, sheep, cattle and deer (Kamalak et al., 2005a) and it plays an important role in the diet of herbivores in areas where there are few or no alternative food sources.

Potential nutritional value was measured using the chemical composition and the gas production technique. Maturation had a negative effect on the chemical composition of the *Arbutus andrachne* leaves. Dry matter and crude protein decreased as maturity increased, while ash and concentrated tannin content increased.

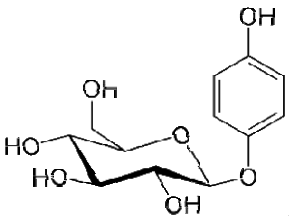
Saker et al. (1991) were the first to describe some chemical components present in *A.andrachne*, namely catechin, epicatechin, and arbutin as well as other components in the tree bark such as monotropin and monidoside. Catechins have antioxidant, anticancer, anti-angiogenic, anti-mutagenic, hypocholesterolemic, anti-aging, anti-diabetic, anti-bacterial and anti-inflammatory effects.

Similarly, Aljabari et al. (2014) conducted research on the preservation and use of *A.andrachne*. They identified the presence of catechin, an antioxidant and simultaneously active flavonoid in leaves of wild plant materials, in callus extract and in *in vitro* cultured tissues of plant origin. HPLC analysis performed for catechin showed 0.063% in callus extract, 2.5% in *in vitro* growing tissues, and 0.5% in wild growth plants.

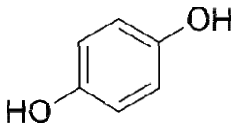
The *Arbutus andrachne* L. tree usually grows in mountainous rocky habitats with alkaline soil, and parts of the plant are known to have valuable medicinal properties due to their extremely high content of antioxidants and some natural pigments that make it a multi-purpose plant. In addition to its medicinal properties, a cosmetic value of dried leaf powder as a skin whitening agent in face masks has been described by Issa et al. (2008) and many other researchers in the field of Cosmetology.

2.2.2 Arbutin

Arbutin (hydroquinone-β-D-glucopyranoside or 4-hydroxyphenyl-β-D-glucopyranoside (Figure 2.2.2.1) is a phenolic glycoside which is naturally synthesized from hydroquinone.



Arbutin



Hydroquinone (quinol)

Figure 2.2.2.1: Chemical structrure of arbutin and hydroquinone

It was initially isolated from the leaves of the *Arctostaphylos sp.* plant (Qiao et al., 2015) but it has also been found in leaves of various other plants in different percentages, in blueberries, cranberries, pears and in the plant families *Lamiaceae*, *Ericaceae*, *Saxifragaceae* and *Rosaceae* (Rychlinska et al., 2012) and it is well known as a substance both for its antibacterial activity that has been observed and for its bleaching action. In fact, in the countries of the East, it is in demand, since it is contained in many beauty products and face creams. Since the 18th century it has been used as an anti-inflammatory and antibacterial agent with application in cystitis, urethritis, pelvicitis and various allergic skin reactions (Thongchai et al. 2007). Arbutin is absorbed from the gastrointestinal tract where it is broken down into hydroquinone aglycone and glucose by the intestinal microflora under the influence of the β-glucosidase enzyme. The antimicrobial activity of arbutin is directly linked to the activity of β-glucosidase (Jurica et al., 2017). In addition, arbutin has been observed to exhibit the ability to relieve cough, dissolve phlegm, and help prevent cougha sthma (Qiao et al., 2015).

Due to its status as a tyrosinase inhibitor, which is involved in the biochemical pathway for the synthesis of melanin from tyrosine (Figure 2.2.2.2), its bleaching properties are summarized in the following: freckle prevention, blotch prevention, sunburn healing, general melanogenesis control. Other substances with a bleaching effect are vitamin C, kojic acid, licorice extract, tobacco root extract, extract from the plant *Scutellaria sp.*, and extract

from berries (Thongchai et al. 2007).

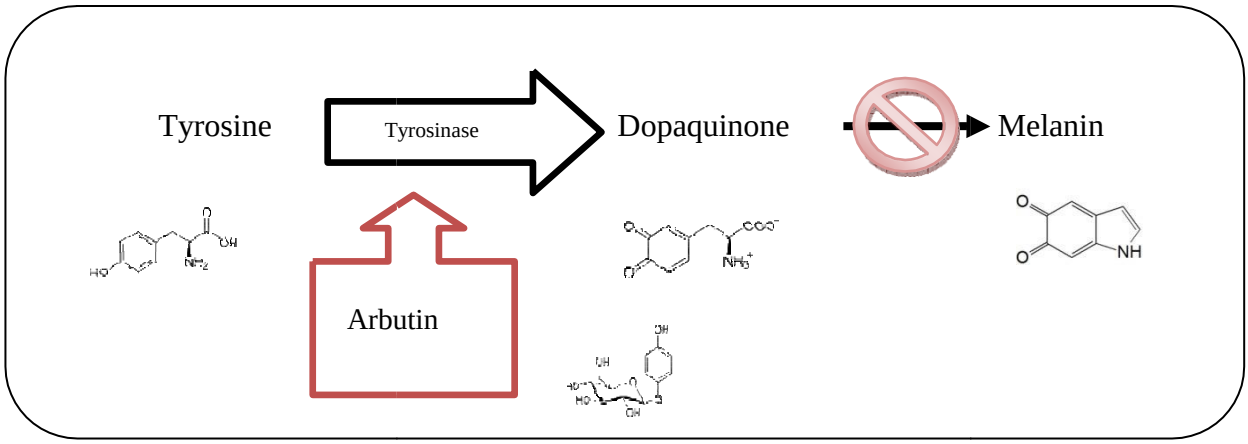


Figure 2.2.2.2: Inhibition of tyrosinase enzyme and eventually inhibition of melanogenesis by arbutin.

In addition to its whitening action, arbutin is a very safe, non-toxic substance, without a bad odor, and acts both as an anti-aging agent and as a UVB/UVC radiation filter (Thongchai et al. 2007).

2.3 Analysis methods

2.3.1 Extractions

By extraction, we mean the process by which a group of compounds or other individual compounds are separated and received from an original material. The methodologies followed are many and certainly differ depending on the species, chemical composition and morphology of the sample, the available media such as solvents and instruments, and the nature of the compounds which is desired to be received.

After many studies and comparisons in the world literature, aimed at determining the best extractant or technique, the conclusion is that it is by no means simple or even possible to design a universal process that would be suitable for the extraction of phenolic compounds for example or any other defined group of compounds of a plant system.

The type of solvent is decisive for the result of the extraction. Some of the solvents used quite often for extractions are: methanol, ethanol, water, acetone, diethyl ether, dichloromethane, toluene, petroleum ether and others.

Increased energy and solvent requirements, as well as the low yield of some conventional methods have led to the search for alternative extractants (Chemat et al., 2012). Several more environmentally friendly solvents are now used and thus the use of petroleum derivatives is avoided. This reduces volatile organic compounds from industry and at the same time avoids other disadvantages such as flammability, unpleasant odors and toxicity. In recent years, "green" methods have been preferred in order to optimize the consumption of the energy used. Some of the "green" methods are: the use of ethanol, the use of methyl esters of fatty acids which can replace petrochemicals, the use of glycerol which can be omitted as a by-product of the esterification of vegetable oils, the use of water as a polar solvent, the use of cyclodextrins, the use of supercritical CO_2 which is a compressed gas at pressures up to 300MPa and temperatures up to 40^oC and can replace organic solvents such as hexane in low molecular weight compounds, the use of microwaves to obtain essential oils, paints and antioxidants, the use of hydrodistillation and the use of ultrasonics (Chemat et al., 2012).

The use of organic solvents has, indeed, greatly decreased, and at the same time the use of water in mixtures with some relatively non-polluting organic solvents has increased, e.g. pure water with the addition of soluble solids that increase yields, such as β-cyclodextrins.

2.3.2 Extractions with β-cyclodextrins

A recent innovation in aqueous extractions is related to the use of cyclodextrins, which carry a hydrophilic outer surface and hydrophobic inner cavity, in aqueous solution for their ability to form inclusion complexes between bioactive compounds and their peculiar hydrophobic cavity through non-covalent bonds. Encapsulation confers some degree of protection to the active compounds against oxidation, evaporation, reaction or migration and at the same time it provides an enhancement of solubility and so these encapsulated or encased components can be used to prepare many enriched foods (Mourtzinos et al., 2011). Cyclodextrins are non-toxic cyclic oligosaccharides, which are derived from the enzymatic degradation of starch and are divided into α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin (Figure 2.3.2.1) with six, seven and eight glucose monomers, respectively (Mourtzinos et al., 2011). Less polar molecules have more access to the system because in this way the energy of the system is reduced, and greater stability is achieved.

In a quite recent research, the extraction of resveratrol and other polyphenols from the ground roots of *Polygonum cuspidatum*, with the help of ultrasound, was effectively carried out in aqueous solution of β-cyclodextrin (1.5%). The selective encapsulation properties of cyclodextrins gave a much clearer extract profile compared to that observed with methanol. Thanks to polyphenol encapsulation, this extract showed excellent water dispersion, higher stability, and antioxidant power (Mantegna et al., 2012).

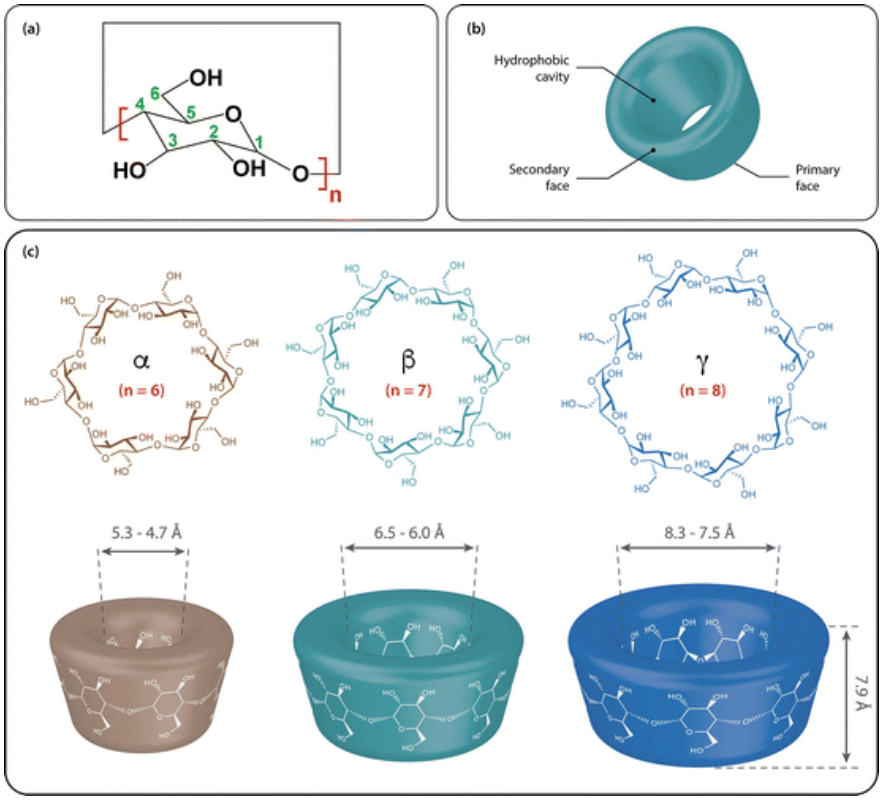


Figure 2.3.2.1: Chemical structures α-, β-, γ-cyclodextrin (Crinietal., 2018)

The advantages of cyclodextrins over other solvents are that they increase the solubility of poorly soluble compounds in water, such as phenolic compounds, increase the stability of compounds that are sensitive to pH, temperatures and radiation, and finally it has been observed that they can reduce or completely mask an unpleasant taste or odor of a component (Diamanti et al., 2017).

2.3.3 High Performance Liquid Chromatography (HPLC)

With liquid chromatography it is possible to separate and quantify polar, non-volatile or heat-sensitive compounds that cannot be analyzed directly with gas chromatography. The highest performance in liquid chromatography is achieved with low flow velocities, which imply a long separation duration. Flow range in liquid chromatography is from 0.5 to 5mL/min. Ordinary columns with 5μm filler operate at a flow of 1mL/min and pressures of 1000-2000psi.

The use of High Performance Liquid Chromatography to determine phenolic substances

is very widespread. Depending on the type of sample and instrument, tests are carried out to obtain the most efficient protocol for each sample. First it must be decided whether the elution will be isocratic, where the composition of the mobile phase does not change during analysis, or gradient where the power of the mobile phase varies achieving better separation.

With isocratic elution, when the sample contains many components, these are difficult to separate, while at the same time the sample components that are strongly retained by the column are eluted very slowly and this results in the expansion of their chromatographic peaks. Gradient elution mixes a patient with a strong solvent in percentages that usually vary over time. Initially, substances that have a short retention time in the column are separated and, with the increase in power, those that are retained for longer are eluted better.

The mobile phase is a mixture of two or more solvents with or without the addition of additives (e.g. buffer solutions). For the selection of the appropriate solvents, their polarity and selectivity must be taken into account. The selection of the mobile phase is made so that its polarity differs from that of the stationary phase so that there is sufficient separation in the components of the sample being analyzed. The selectivity of the solvent depends on its ability to act as proton acceptor, proton donor, or with strong bipolar moments in relation to the other substances.

A characteristic instrument arrangement is shown in Figure 2.3.3.1 below:

Figure 2.3.3.1: Schematic representation of an HPLC instrument assembly.

The choice of the column, and therefore of the stationary phase, is also very important, since it matters which compounds each can retain and which will allow them to be eluted, always according to the substance to be determined. These

solvents also play a very important role since they are the ones that retain and drift the substance of interest to us and for the best result it is advisable to test with different solvent ratios.

In the literature for the determination of phenolics, one or more, C8 or C18 columns are used, most reports opted for gradient elution due to better results, but there were also many that obtained satisfactory results with isocratic elution and generally agreed on temperatures, wavelength (nm) of the UV-Vis probe, flow velocity and solvents, with most studies opting for water to methanol or acetonitrile ratios and the addition of a very small percentage of an acid for clearer and more acute chromatographic peaks.

Each sample, depending on the nature of the sample, needs specific processing before entering the column. Below, in Tables 2.3.3.1 and 2.3.3.2, some protocols which were used as a basis for the creation of a new protocol that meets the needs of the specific sample are presented.

Table 2.3.3.1: Protocols for HPLC in honeys for the determination of phenolic components

Source	Colu mn	Elution	Solvents	Flow velocity	Injection volume	Probe	nm
Fuertes et al. 2019	C18	Gradient	Water with 1% FormicAcid-Acetonitrile	1mL/min	20μL	UV	280, 320, 340, 360
Ayvaz et al. 2018	C18 (30^{o} C)	Gradient	2% Acetic acid inWater-70% Acetonitrile in Methanol	1.2mL/min	25μL	UV	280-315
Nascimento et al. 2018	C18	Gradient	Formic acid-Methanol	0.2-0.8mL/min	5μL	UV	260 ή 320
Vasic et al. 2019	C18	Gradient	Water with 0.1% Acetic Acid & 100% Acetonitrile	0.3mL/min	5μL	MS	-

Table 2.3.3.2: Various protocols for HPLC in plant extracts for the determination of arbutin

Source	Column	Elution	Solvents	Flow velocity	Injection volume	Probe	nm	Standard curve concentrations	Time
Parejo et al. 2001	C8	Isocratic	95:5 Water:Methanol	1mL/ min	10µL	UV	280	25-400µg/mL	3.7 min
Thongchai et al. 2007	C18	Isocratic	89:10:1 Water:Methanol: HCl 0.1M	1mL/ min	100µL	UV	222	(1000µg/mL stock solution) 0.5-30µg/mL	5.7 min
Jeon et al. 2015	C18	Gradient	Water- Methanol	1mL/ min	10µL	UV	280	12.5-250µg/mL	7.1 min
Qiao et al. 2015	C18 ($30^{o}C$)	Gradient	Water- Methanol	1mL/ min	10µL	UV	220	$1*10^{-5}$-0.25 mg/mL	2 min
Wang et al. 2015	C18 ($45^{o}C$)	Gradient	Water-Methanol-Acetic acid 0.1% (v/v)	0.5mL/min	10µL	UV	280	5-500µg/mL	9.68 min
Rychlinska & Nowak 2012	RP18	Gradient	0.5% Aqueous solution of Orthophosphoric acid-Methanol	1mL/ min	20µL	UV	289	7.5-500µg/mL	3.36 min

More specifically, arbutin was reported bibliographically to have various retention times depending on the method, the solvents, the column, and the organ. In some researches it had a retention time of close to 3min, while in others it reached 10min. Below (Figure 2.3.3.1) some characteristic chromatograms of arbutin (glycoside) and hydroquinone (aglycone) on different plants species are shown. The peak at 3.3min is of arbutin and the peak at 3.9min is of hydroquinone.

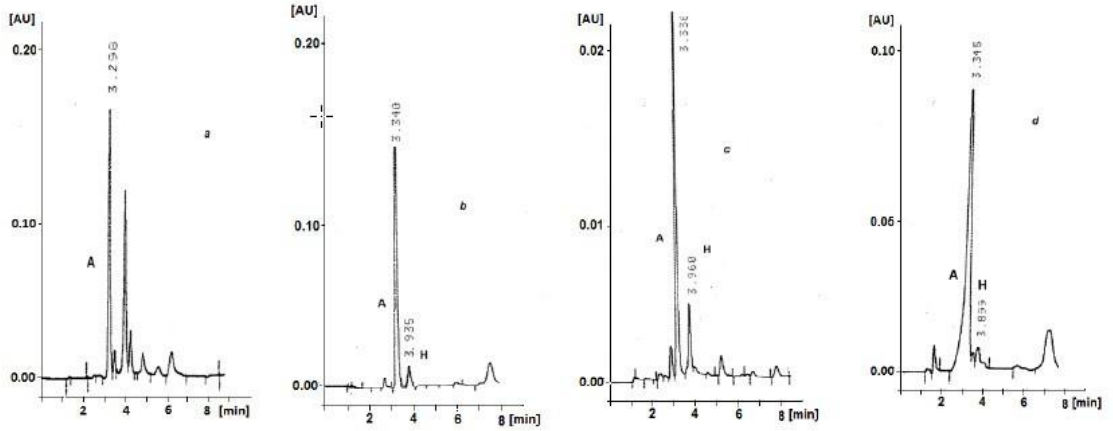


Figure 2.3.3.1: HPLC chromatogram of arbutin: a. aqueous extract of *B. crassifolia* b. *A. uva-ursi* c.*Majorana bortensis* d. methanolic extract A. *uva-ursi* (Rychlinska et al., 2012).

2.4 Enrichments

In the world literature there were not many reports on enriched honeys, which, on the one hand, shows that it is a new proposal that has not been tested enough yet and, on the other hand, it is an attempt to satisfy the consumer public, since various honey enrichment ideas have been tested, even at an initial or approximate stage. Some of them are: honey enrichment with methylglyoxal (MGO), an agent that is found almost exclusively or at least in very high concentrations in Manuka honey and has been observed to have particular

antibacterial properties, honey enrichment with prunes in various concentrations in order to increase its antioxidant activity, enrichment with various beekeeping products such as royal jelly, pollen, propolis, enrichment with *Rosa spp.* fruit, enrichment with herbs.

In the Jervis-Bardy et al. (2011) experiment, Manuka honey (790mg/kg MGO), non-MGO honey supplemented with 790mg/kg MGO and plain MGO solutions were used for comparisons. The experiment was then repeated using non-MGO honey solutions supplemented with sufficient MGO to achieve concentrations exceeding those observed in commercially available Manuka honey preparations. The aim of the experiment was to observe the reaction of strains of *Staphylococcus aureus* in the presence of the above honeys, enriched or not. Manuka honeys, with the specific amounts of MGO, showed bactericidal activity, while honeys without MGO did not show bactericidal activity. Solutions with MGO had a corresponding effect only at concentrations >1.05mg/mL MGO. In general, they observed that honeys either with MGO naturally (Manuka), or non-MGO ones in which it was artificially added had much better results than solutions containing only MGO. Finally, they concluded that methylglyoxal is only partially responsible as a substance for the antibacterial activity of honey.

Tumbas et al. (2012) tried to enhance the antioxidant activity, phenolic content and flavonoids of acacia honey by adding pieces of fruit to the honey, more specifically prunes cut in four and in percentages of 20%, 30% and 40%. The addition of prunes by 40% showed to increase the total phenolic content by 2.5 times and the total flavonoids by 11.5 times, compared to the control that was non-enriched acacia honey (Figure 2.4.1).

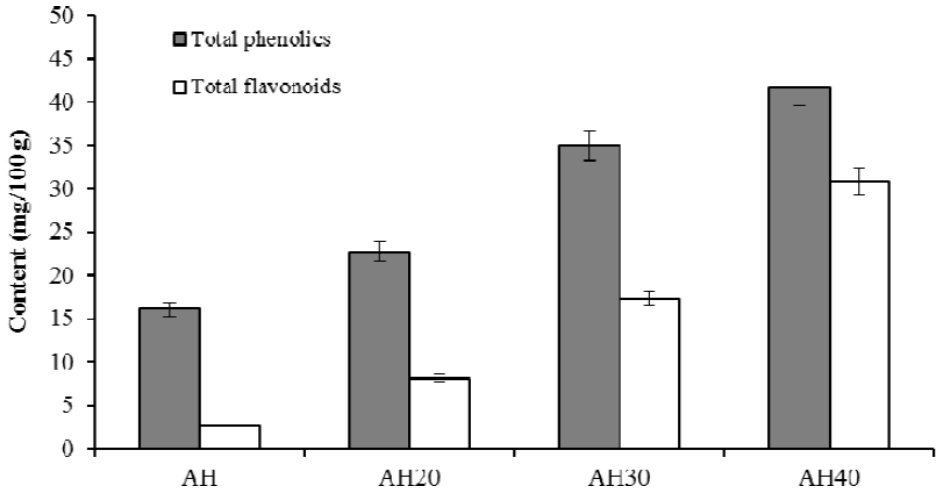


Figure 2.4.1: Concentrations of total phenols and flavonoids in acacia honey enriched with pieces of prune at 20, 30, 40 %. (Tumbas et al. 2012)

Similar results were observed in the determination of antioxidant activity with DPPH, where the enriched samples had up to 10 times greater antioxidant activity than the control. In all tests, the increase was proportional to the amount of prunes added each time. Nevertheless, the aroma and colour of the enriched acacia honeys were the ones that received the lowest scores in the organoleptic test, while the taste was not particularly affected, making it marginally acceptable.

The research of Juszczak et al. (2015), who tried to enrich classical blossom honeys with beekeeping products produced by the same apiary, also seems to be of interest. They tried enrichment with royal jelly, pollen, propolis and the so-called "beebread" (Figure 2.4.2), which is essentially pollen along with nectar, bee enzymes and honey. The largest differences were observed (Table 2.4.1) in the enrichment with propolis and "beebread", where the values of total phenolics, total flavonoids and antioxidant activity seemed to be quite high, with higher those of "beebread", although it is reported that perhaps enrichment with propolis is practically easier since it only needs a very small amount of enrichment for maximum results and therefore benefits.

Table 2.4.1: Total phenolics, total flavonoids and antioxidant activity of enriched blossom honeys with beekeeping products

Sample (n = 5)	Total phenolics (mg GAE/100 g)	Total flavonoids (mg QE/100 g)	Antioxidant activity (DPPH*)(%)
Blossom Honey (A)	36.06± 10.18	4.48± 1.69	12.96± 3.62
A + royal jelly	39.88± 7.11	3.48± 0.83	13.06± 3.17
A + pollen	84.22± 23.84	11.09± 2.11	22. 9± 12.92
A + beebread (pollen+nectar+enzymes+honey)	179.96 ± 127.57	23.68± 13.42	41.19± 24.35
A + propolis	114.82± 42.5	15.96± 6.38	34.08± 9.73

(Juszczak et al., 2015)

Figure 2.4.2: «beebread»

Stajner et al. (2014) tried enriching acacia honey (*Robinia pseudoacacacia)*) with components from the *Rosa canina* plant (Figure 2.4.3-5). They received fruits from the plant, removed the seeds internally, mashed it and added it to honey in two different concentrations: 5g/100g and 10g/100g. They resulted in the selection of fruits as a part of the plant to be enriched after surveys that agreed that it is the part of the plant with the highest percentage of vitamin C and with the highest antioxidant activity. They performed various measurements:

determination of antioxidant activity with various methods, total phenolics, ascorbic acid percentage, colour measurement, measurement of fatty acid oxidation.

Figure 2.4.3-5: (from left to right): *Rosa canina* fruit, acacia honey, *Robinia pseudoacacia* blossoms.

They concluded that the enriched honey with the highest concentration (10g/100g), showed the best properties, ie particularly increased antioxidant activity (Figure 2.4.6) compared to the control, high percentage of phenolic acids as well as high percentage of vitamin C.

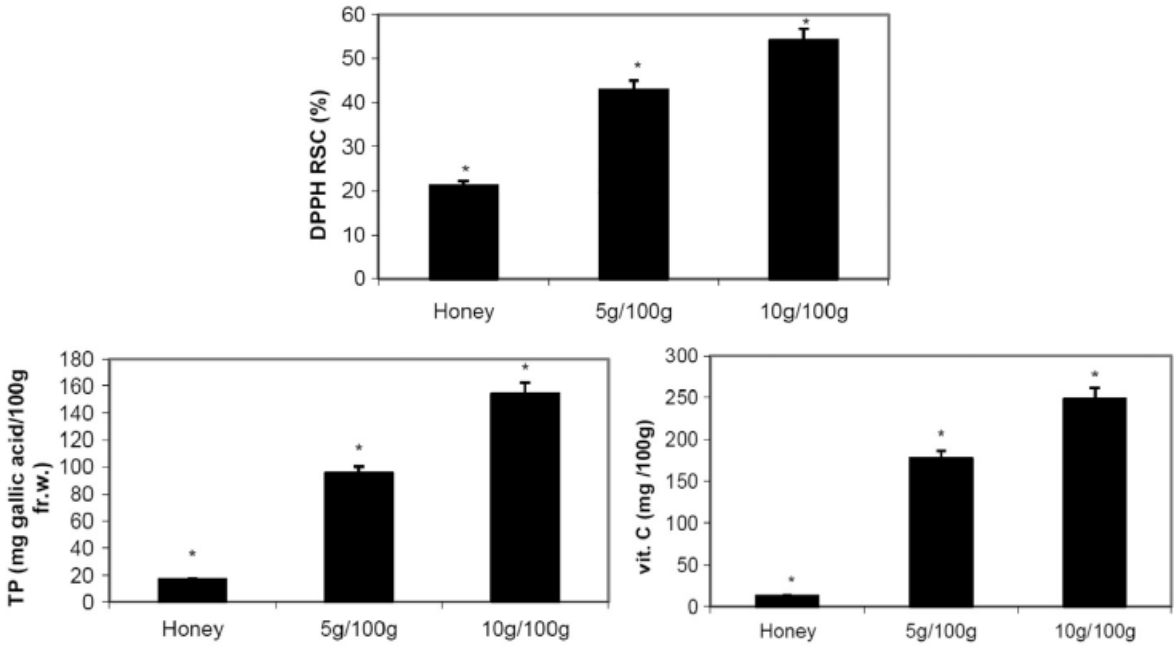


Figure 2.4.6: Acacia honey antioxidant activity, total phenolic content and ascorbic acid concentration, in relation to acacia honey enriched with 5g/100g and10g/100g fruit of *Rosa spp.* (Stajner et al. 2014)

The study of Dzugan et al. (2016) focused on the determination of chemical characteristics of honeys after herbal enrichment in three different ways. They divided the honeys into three groups:

1. Commercial honeys collected from bees fed syrup with herbal/plant extracts (according to the patented APICENTRUM ZN-Z (*) / 88 recipe). More specifically, it was used: nettle (*Urtica* L.), nutmeg (*Crataegus* L.), pine (*Pinus* L.), aronia (*Aronia* Medik.) and aloe (*Aloë* L.)
2. Natural honeys resulting from the collection of nectar from plants such as nettle (*Urtica* L.), blackberry (*Rubus* L.) and aronia (*Aronia* Medik.).
3. Fine crystallized honey with the addition of dried herbs (1.5%w/w) such as lavender blossoms (*Lavandula* L.), lemon balm leaves (*Melissa* L.), nettle (*Urtica* L.) , mint leaves (*Mentha* L.) and ginger root (*Zingiber Boehm*.).

This study confirmed that the groups of apiculture products selected to be enriched with herbs, extracts or other parts of a plant show very different physicochemical properties and antioxidant activity compared to the control which was blossom honey without any intervention from the same area. However, all samples studied were enriched according to the

requirements for natural honey. Compared to classical blossom honeys, better antioxidant activity and higher phenolic content appeared in fine crystallized (creamy) honeys to which dried herbs were added, and appeared to be much superior to herbal honeys in terms of these parameters. Herbal honey, however, has a consistency similar to natural liquid honey and is therefore preferable and more attractive to consumers. The controlled feeding of the bees with the plant syrup allowed the introduction of the desired plant bioactive components into the honey. Also, the level of enrichment of each honey depends on the plant additive used since the various plant species produce various secondary metabolites that show antioxidant activity, and on the very nature of each honey which depends largely on the origin among other things. Finally, the fortification of honey with herbs or plant material allows the creation of new, innovative products with modified chemical composition and improved antioxidant activity, compared to the control, i.e. raw honey, which can have a positive impact on human health.

Chapter 3 - Materials & Methods

3.1 Materials

3.1.1 Samothrace honey samples

The honey samples came from an apiary in Samothrace, some of which were from earlier harvests (2016-2018), of the same apiary, and some from the first (A΄) and second (B΄) honey harvests of 2019. Upon receipt of the samples, they were stored in a dark and cool place and remained there stored throughout the experimental process. A total of 15 honeys were analyzed: 14 Samothrace honeys and one Manuka MGO 30+ honey (Manuka Health Honey MGO 30+), as a positive control. One of the 14 Samothrace honeys was used as a honey base for honey enrichments.

Table 3.1.1.1: Honey samples

INITIAL CODIFICATION	FINAL CODIFICATION	HONEY ORIGIN YEAR
S	**1**	**Samothrace (2016)**
S4	**2**	**Samothrace (2018)**
S5	**3**	**Samothrace (2018)**
M	**4**	**Manuka (New zealand)**
1.2	**5**	**Samothrace (2017)**
1.3	**6**	**Samothrace (2017)**
3.3	**7**	**Samothrace (2017)**
9.1	**8**	**Samothrace (2019 A΄)**
9.2	**9**	**Samothrace (2019 A΄)**
9.3	**10**	**Samothrace (2019 A΄)**
9.4	**11**	**Samothrace (2019 A΄)**
9.5	**12**	**Samothrace (2019 A΄)**
9.6	**13**	**Samothrace (2019 A΄)**
9.7	**14**	**Samothrace (2019 A΄)**
9.8	**15**	**Samothrace (2019 B΄)**

Table 3.1.1.1 shows the codes of the samples studied. Apart from the control, namely Manuka honey with code 4 (positive control), the other codes all correspond to Samothrace honey.

3.1.2 Samples from the *Arbutus andrachne* plant

The plant samples: young leaves (Y), ripe leaves (R), over-ripe/aged leaves (R2), fruits (F), as well as blossoms (B) were also collected from Samothrace, from the same area as the apiary from which all the honey samples came, at four different times.

Table 3.1.2.1 shows the codes of the samples from the different parts of the plant studied and compared.

Table 4.1.2.1: Samples of the *Arbutus andrachne* plant

CODIFICATION	PART OF THE PLANT
B	Blossoms
F	Fruits
Y	Young leaves
R	Ripe leaves
R2	Over-ripe leaves

The various plant parts were dried (at 40^{o}C for 24 hours), pulverized and then the samples F, Y, R were sieved with 5 sieves of different cross-sections so that they could be divided into fractions. The selection of fractions for extraction would then be done from these fractions, since grain size is very important in the extraction process, time and yield.

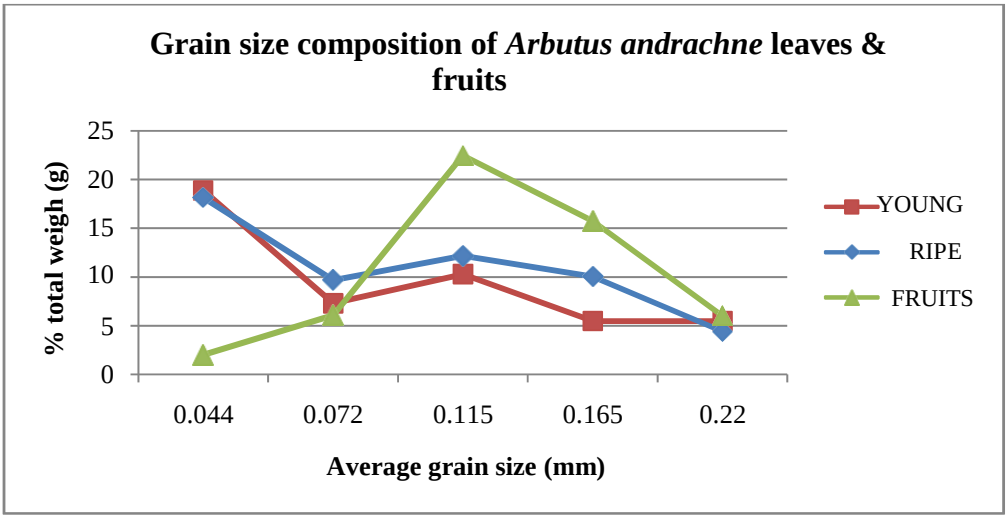


Diagram 3.1.2.1: Grain size composition of leaves and fruits of the *Arbutus andrachne* plant.

The first fraction of the sieve (DIN 16) with a grain cross-section of 0.24mm, which was the largest in mass, as parts of the ribs and stem of the leaves were retained there as well as hard parts of the fruit, which could not be satisfactorily pulverized, was rejected by the upcoming extraction process. The remaining fractions (Y, R, F) were used in the extractions, so the average grain size finally used is 0.12mm.

3.2 Methods

3.2.1.1 Extractions with organic solvents

For the extraction process, various tests were performed both with extractive agents and with percentages of dry matter and extraction time. In an experiment performed with gradual measurements at the 1st hour of extraction , 2nd hour, 4th hour, 8th hour and after 24 hours of extraction, no statistically significant difference was observed, so the extraction time for this plant material was stabilized at one hour of extraction at ambient temperature after shaking. According to the protocol of Parejo et al. (2001), the extractions were carried out using 0.5g of pulverized plant material in 25mL of solvent, at ambient temperature. Regarding the solvent, various tests were performed, some of them with different percentages of methanol-water, ethanol-water, methanol-ethanol, and distilled water. The yield of the extracts was measured based on their antioxidant capacity. Methanol-water extracts showed little yield compared to

ethanol-water extracts and they were discarded. The best yield was observed in the extract with 80 ethanol: 20 water (Diagram 3.2.1.1.), which was finally decided to be used for the determination of antioxidant capacity, total phenolic acids, and total flavonoids. The different letters indicate a statistically significant difference.

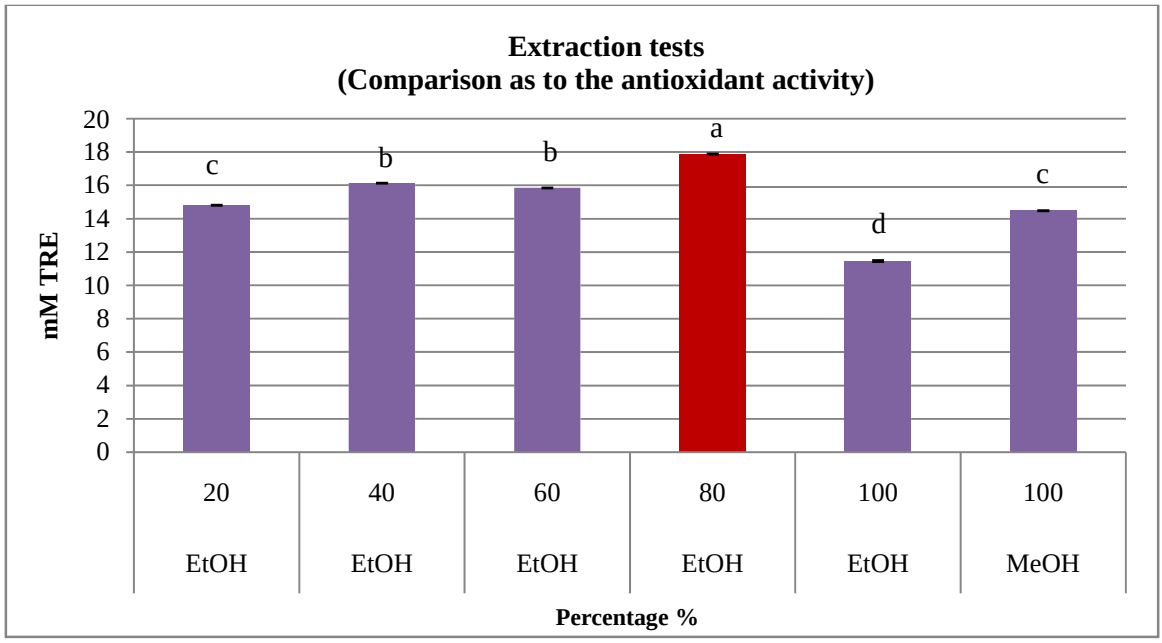


Diagram 3.2.1.1.: Comparison of antioxidant activity of leaf extracts of the *A.andrachne* plant in various percentages of solvents as extractive agents, with a significance level of $P< 0.05$.

3.2.1.2 Extractions with water and β-cyclodextrin

It was considered appropriate to perform an extraction test with both water and β-cyclodextrins, since the purpose of the experiment is the enrichment of a food product (Samothrace honey). B-cyclodextrins act as a different encapsulating agent and exhibit greater yields than other, common, extracting agents, as shown in section 2.3.2. Since it would not be possible to add an extract containing an organic solvent to the food, regardless of whether these were used due to their high yields for the determination of chemical

agents, enrichment tests will be performed with a solvent that can be consumed.

The water extraction followed the same protocol as the organic solvent extractions, according to Parejo et al. (2001), using 0.5g of pulverized plant material in 25mL of solvent, at ambient temperature for one hour.

For the plant extract of cyclodextrins, 2.775g of β-cyclodextrin was added to 150mL of distilled water, which is the maximum amount of β- cyclodextrin, until it is completely dissolved. 10g of dry matter was then added and left in a magnetic stirrer for two hours of extraction. Finally, vacuum filtration took place, receiving the pure extract and storing it in the freezer.

3.2.2. Chemical analyses

3.2.2.1 Determination of antioxidant capacity

For the determination of antioxidant capacity, it was used the 1.1-biphenyl-2-picryl-hydrazylth free radical (DPPH*) binding method. After appropriate dilutions so that the instrument readings are within the reference curve, 75μL of sample were mixed with 2925μL of DPPH* reagent (100μM in methanol) (TCI Co., Japan). The mixing was done with vortex and then the sample was left to react for 30 minutes in a dark place. The absorption of the samples was measured at 515nm using cells (1cm Acrylic cuvettes, BrandTech Scientific Inc., Essex, U.K.) in UV-Vis spectrophotometer (UV-1800, Shimadzu Co., Japan). At the same time, the absorption of the control containing only DPPH* reagent was measured, without the addition of a sample. The results are expressed in mM Trolox Equivalents (TRE) (Trolox vitamin E analogue with the ability to bind the free radical DPPH*) , through the equation:

$$\mathbf{AA\ (mM\ TRE) = 0.016 - \%SA * 0.034}$$

which resulted from linear regression, after correlation of the %SA of the Trolox reagent (Sigma-Aldrich Chemie GmbH, Germany) and its concentration (0.1-1.6 mM Trolox) with R^2=0.998, where:

%SA = (A_{blank}-A_{sample})/A_{blank} * 100.

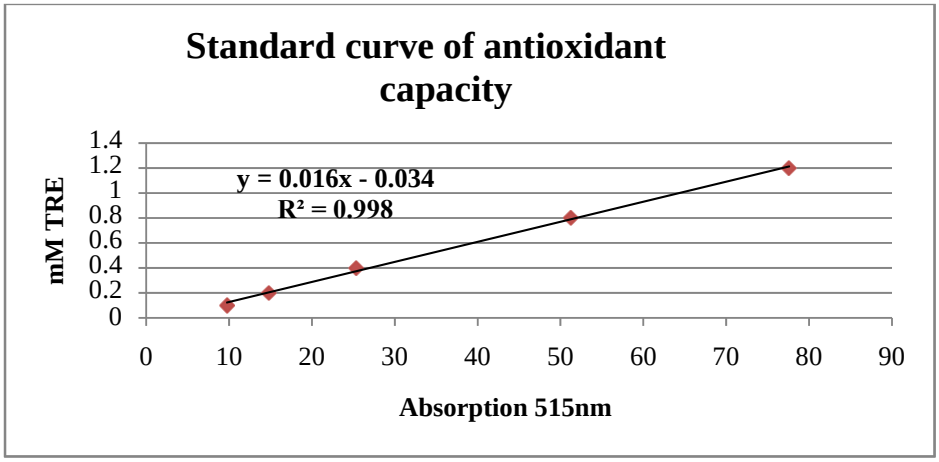


Diagram 3.2.2.1: Standard curve of antioxidant capacity (Trolox equivalents)

3.2.2.2 Determination of total phenolic compounds

For the determination of total phenolic compounds it was used the Folin - Ciocalteau method. Initially, 2370μL of distilled water is mixed with 30μL of extract properly diluted to keep the indications within the standard curve. After shaking, 150μL of Folin – Ciocalteau reagent are added. Stirring follows again and after 1min 450μL of saturated sodium carbonate solution (Na2CO3 20% w/v) are added. The mixture is shaken and stored in a dark place at room temperature for 2 hours. The reaction product is measured at 750nm. In the blank sample prepared for zeroing it is placed methanol in the place of the sample. The concentration of polyphenols in the sample is calculated using a Gallic acid reference curve and expressed as gallic acid equivalents via the equation with R^2=0.995:

$$\textbf{TPC (mg GAE/L)} = 983.9 * A_{Sample} - 105.7$$

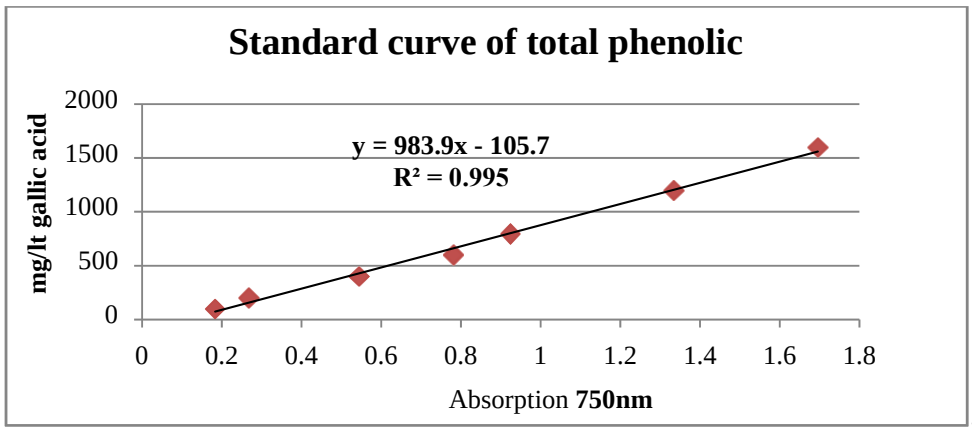


Diagram 3.2.2.2: Standard curve of total phenolic compounds (Gallic acid equivalents)

3.2.2.3 Determination of total flavonoids

For the determination of total flavonoids, initially 120μL of AlCl3 solution (2% AlCl3 in methanol/acetic acid mixture, 95/5, v/v) and 1680μL of 5% methanolic acetic acid solution are added successively in 1.2mL of extract of suitable dilution. The absorption of the formed complex is measured at 415nm with reference solution after 30min left at ambient temperature to react. Correction of the absorption values is performed by removing the initial absorption (415nm) of the corresponding solution in the reaction environment in the absence of the reagent. The results are expressed in mM of quercetin extract through the equation with R^2=0.991:

$$\textbf{TFC (mM quercetin)} = 0.854 * A_{Sample} - 0.101$$

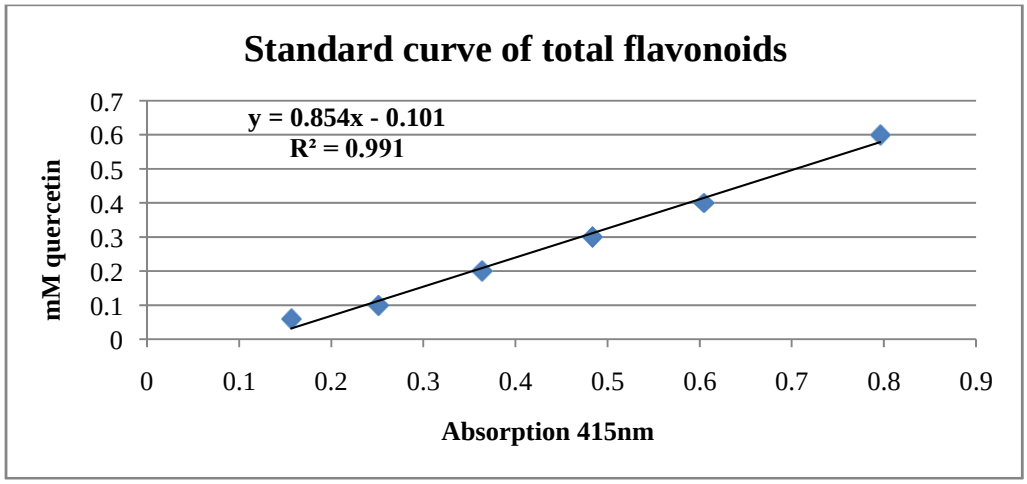


Diagram 3.2.2.3: Standard curve of total flavonoid compounds (quercetin equivalents)

The chemical analyses (antioxidant capacity, total phenolic acids, total flavonoids) carried out for the plant extracts were carried out in exactly the same way, with different dilutions, and in the honey samples as aqueous solutions.

3.2.2.4 Arbutin analysis with High Performance Liquid Chromatography

The purpose of the High Performance Liquid Chromatography is both the qualitative and subsequently the quantitative determination of the percentage of arbutin in plant extracts, in honeys and finally in enriched honeys. Based on the protocols of Parejo et al. (2001), Jeon et al. (2015), Wang et al. (2015), Thongchai et al. (2007), initially some tests with different solvent ratios were performed.

The preparation of plant samples was done according to Parejo et al. (2001). dried plant powder, 50mg, addition to 25mL of solvent (95:5 HPLC water:HPLC methanol) at 25^{o}C for 30 minutes. It was then centrifuged at 7600g for 10 minutes and the supernatant was collected for analysis.

To prepare the honey samples, based on the studies of Vasic et al. (2019), 2.5g of honey was initially added to 10mL of HPLC water (ultrapure) followed by immersion in an ultrasonic bath for 15 minutes aiming at

the homogeneity of the sample. Then, transfer to a 25mL volumetric flask and fill with HPLC water to the mark was carried out. Finally, filtering was carried out with a 0.45μm PTFE membrane filter.

A new protocol emerged, using a C18 column and isocratic elution with 95:5 solvents of HPLC water:HPLC methanol with the addition of 0.1% acetic acid. The flow rate selected was 1mL/min and the volume of each infusion was 20μL. A UV-Vis detector was used as a detector and the photometry was carried out at 280nm. The whole process took place at 30^{o}C. The standard arbutin curve was created with different standard arbutin concentrations (Arbutin 98+ %, Alfa Aesar, Kandel, Germany) ranging from 5μg/mL to 100μg/mL and the resulting equation with R^2= 0.999 is:

Arbutin (μg/ml)=3*10^{-5}*Area-0.649

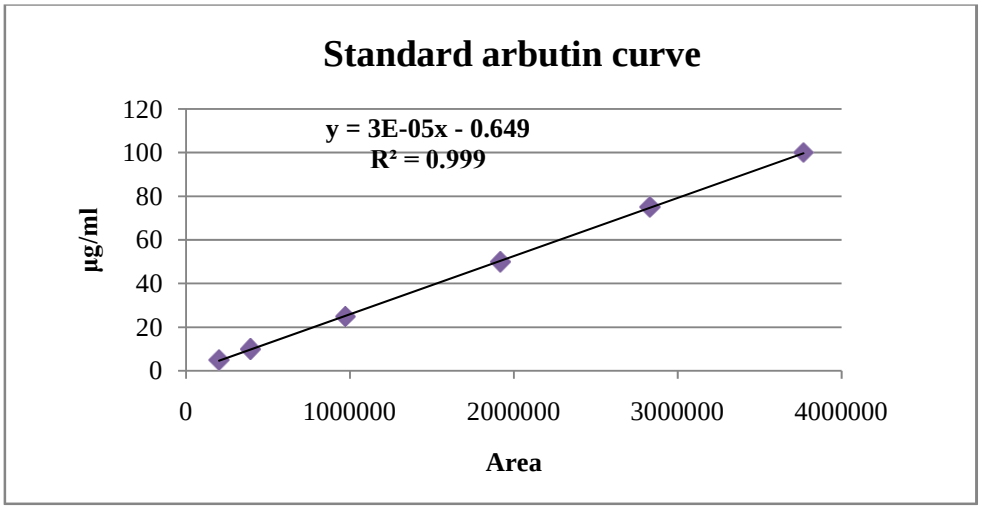


Diagram 3.2.2.4: Standard arbutin curve

Retention time at 1620 PSI was 6.95min.

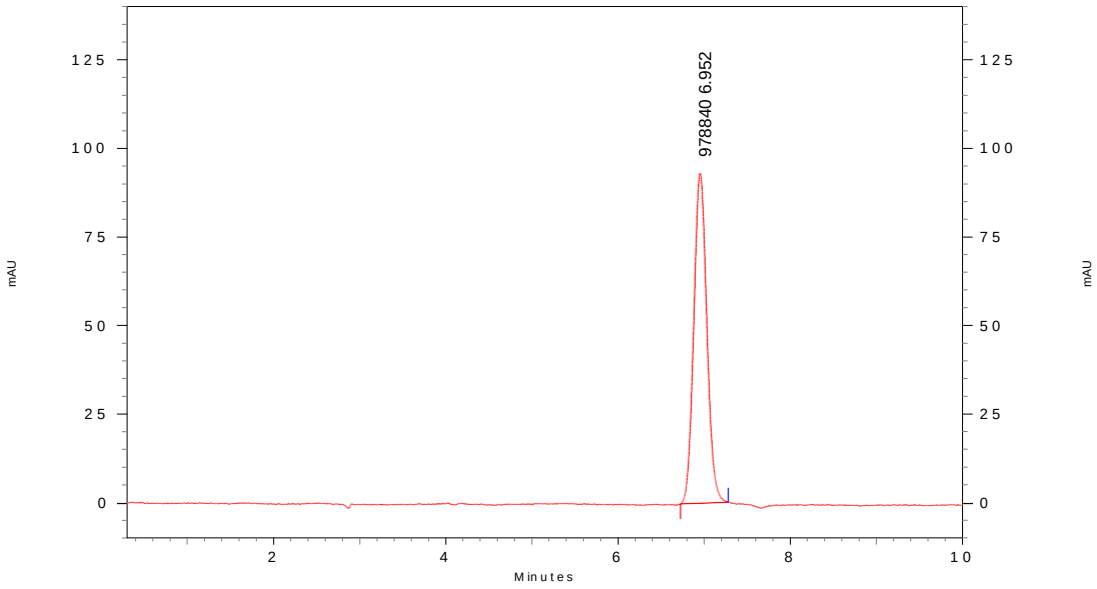


Diagram 3.2.2.5.: 25μg/mg arbutin standard chromatogram

3.2.3 Enrichments of honeys

In order the enrichments of honeys to be possible, extractions (Section 3.2.1.2) were initially carried out in an aqueous medium in two ways: aqueous extractions and extractions with β-cyclodextrin at ambient temperature. After centrifugation and filtration the extracts were stored in the freezer.

The Samothrace honey (Sample 1) which had relatively low values of antioxidant activity, phenolic acids and flavonoids compared to the other Samothrace honeys was chosen as the enrichment basis.

The enrichment rates were selected taking into account the Council Directive 2001/110/EC of the EU on moisture in honey, which should not exceed 20% for most honeys and 23% for heather honey. In case this percentage is exceeded, problems are created in honey from the development of yeasts. The percentages selected for enrichment were 2.5% and 5% in aqueous extract of young and over-ripe leaves of the *A. andrachne* plant. The initial moisture of the honey samples averaged 17% and after the highest enrichment rate, the moisture averaged 21%.

The mixing was carried out with Ultra-Turrax at a very low speed for a few seconds, so as not to raise the temperature of the enriched honey too much, while at the same time it was kept in a container with water and ice, for better control of the sample temperature.

10 enriched sample combinations were created (Table 3.2.4.1): Honey1 with 2.5% and 5% extract of over-ripe leaves with β-cyclodextrin, Honey 1 with 2.5% and 5% extract of young leaves with β-cyclodextrin, Honey 1 with 2.5% and 5% aqueous extract of over-ripe leaves, Honey 1 with 2.5% and 5% aqueous extract of young leaves, Honey 1 with 2.5% and 5% water (control). The enriched honeys were stored in the refrigerator and their content of phenolic acids, flavonoids, antioxidant activity and antibacterial activity were chemically determined, using the same protocols used for the determination of the remaining non-enriched honey samples.

Table 3.2.4.1 shows the codes of the samples that participated in the experimental enrichment process.

Table 3.2.4.1: Samples of Enriched Honey

INITIAL CODIFICATION	FINAL CODIFICATION	ENRICHMENT
S β-CD R2 5%	**16**	Honey 1 + 5% extr. R2 (over-ripe leaves) with β-CD
S β-CD Y 5%	**17**	Honey 1 + 5% extr. Y (young leaves) with β-CD
S β-CD R2 2.5%	**18**	Honey 1 + 2.5% extr. R2 with β-CD
S β-CD Y 2.5%	**19**	Honey 1 + 2.5% extr. Y with β-CD
S R2 5%	**20**	Honey 1 + 5% extr. R2
S R2 2.5%	**21**	Honey 1 + 5% extr. Y
S Y 5%	**22**	Honey 1 + 2.5% extr. R2
S Y 2.5%	**23**	Honey 1 + 2.5% extr. Y
S H_2O 5%	**24**	Honey 1 + 5% H_2O
S H_2O 2.5%	**25**	Honey 1 + 2.5% H_2O

3.2.4 Microbiological tests

3.2.4.1 Antibacterial activity (Agar Well Diffusion Assay)

The diffusion technique through Agar Well Diffusion Assay was followed according to Anthimidou & Mossialos (2012) and Stagos et al. (2018). The honey samples tested were thick, diluted with PBS 1:1 (Phosphate Buffered Saline, pH 7.1), processed with catalase, to determine whether the antibacterial activity is due to H_2O_2 and enriched with *A. andrachne* leaf extracts (with β-cyclodextrin and water). Microbiological tests were performed at the Laboratory of Microbiology and Food Hygiene.

The antibacterial activity of the samples was tested against three pathogenic bacteria: *Escherichia coli* (O:44 NCTC9702, National Collection of Type Cultures, AFRC, Reading, England), which is gram-negative rod-shaped, *Staphylococcus aureus* (NCTC6751), which is gram-positive sphere-shaped, and *Listeria monocytogenes* (Scott A), which is gram-positive rod-shaped.

Bacteria (*E.coli, L.monocytogenes, S.aureus*) were kept frozen (-80^{o}C) and were activated by two consecutive recultures in Brain Heart Infusion Broth (Pronadisa, Spain) and incubation at 37^{o}C for 24 hours. They were then centrifuged (8000rpm, 10min), followed by washing the cells by suspending them in saline. Then they were centrifuged again and finally a cell suspension in new saline was created, with a turbidity of 0.5 on the McFarland scale (corresponding to $1.5*10^8$ (cfu/mL). A decimal dilution followed and 0.1ml ($1.5*10^6$cfu) was coated on a surface of a 15ml solidified Mueller-Hinton Agar II substrate (Pronadisa, Spain). This was followed by the creation of the wells, with a diameter of 5mm, using the wide spout of a sterile pasteur pipette. In each well, 100mg of each sample were placed, ie insoluble honey, honey diluted with PBS 1:1, processed honey with catalase solution 1:1 and the sample Honey 1 enriched with extract of the *A. andrachne* plant (extraction with water and β- cyclodextrin) in 2.5% and 5%.

The plates were initially left at 4^{o}C for 2 hours in order the diffusion to be made and then the incubation followed at 37^{o}C for 18-20 hours. In the presence of antibacterial activity, a clear zone is observed around the well where the sample was placed, due to

the inhibition of the growth of the bacterium. Finally, the diameter of zone of inhibition was measured and recorded, including the diameter of the well.

Two artificial honeys were also prepared as controls in the laboratory, each consisting of 1.5g sucrose (D-saccharose, Reidel-deHaen, Germany), 7.5g maltose (Maltose (Monohydrat) krist., Merck), 40.05g fructose (D-fructose, Panreac, Spain), 33.5g glucose (D-glucosemonohydrate, DuchefaBiochemie, TheNetherlands) in 17g water (sample 27-negative control) or in 17g aqueous extract of over-ripe *A. andrachne* leaves (sample 26-positive control). Manuka honey (MGO 30+), of New Zealand origin, known for its antibacterial activity, was also used as a control.

3.2.4.2 Processing with catalase

The samples were processed with catalase solution (0.02g catalase in 10mL PBS) in order to investigate whether the antibacterial activity of the samples is due to H_2O_2. They were only tested against *S. aureus*, as it was the species against which they showed the strongest inhibition. (Snow & Manley-Harris, 2004).

3.2.5 Statistical analysis

Statistical analysis of the results was performed with the help of the IBM SPSS Statistics version 23 for Windows (IBM Corp.). In all analyses, one-way ANOVA and Tukey HSD post-hoc test was performed with a statistical significance level of $P< 0.05$.

Chapter 4 – Results & Discussion

4.1. Chemical analyses

4.1.1. Samothrace Honeys

From the analysis of total phenolics (Figure 4.1.1.1), it was shown that Samothrace honeys generally have quite high concentrations of phenolic acids, with the highest of all, ~880mgGAE/L, belonging to honey 12. The lowest concentration of phenolic acids, ~345mgGAE/L, was found in honey 1, which is the honey selected for enrichment in the subsequent experimental process, followed by Manuka honey with a phenolic acid concentration of 349mgGAE/L.

The following diagrams present the concentrations and the statistically significant differences between Samothrace honey samples, Total Phenolics, Antioxidant Capacity (DPPH) and Total Flavonoids, with a significance level of $P<0.05$ based on the Tukey post hoc test. The lack of a common letter indicates statistically significant differences.

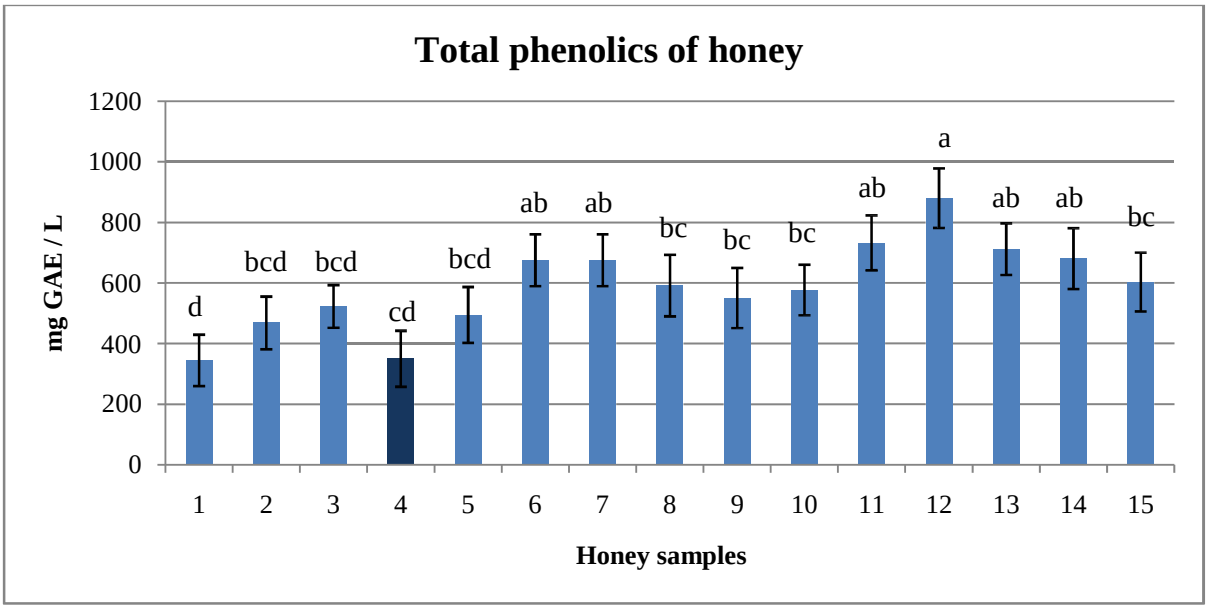


Diagram 4.1.1.1: Differences in total phenolics of Samothrace honey and Manuka honey (4), with a significance level of $P< 0.05$.

The antioxidant capacity, as shown in Diagram 4.1.1.2 below, of Samothrace honeys was found to be particularly high with the highest concentrations belonging to samples 2, 5,

6, 7 and reaching ~2.87mMTRE, while the Manuka honey control was found to have a concentration of ~1.25mMTRE. Although the antioxidant capacity diagrams quite often follow the phenolic acid diagrams, in this case this does not seem to be the case, which shows that the antioxidant capacity is due to chemicals other than phenolic acids.

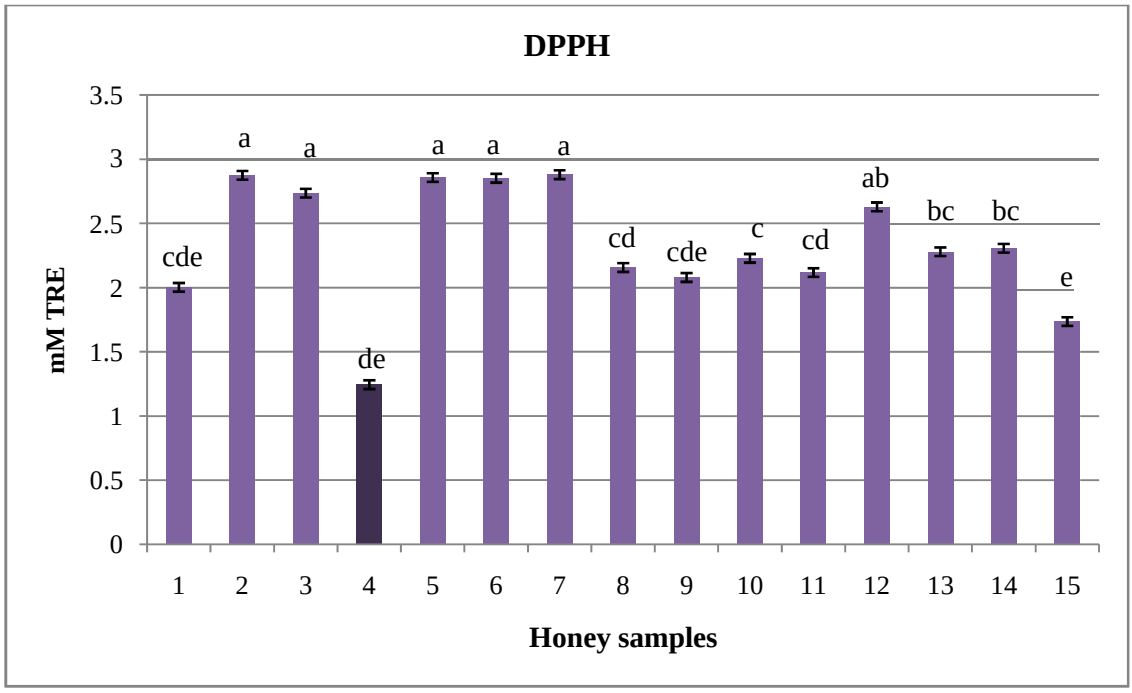


Diagram 4.1.1.2: Differences in antioxidant capacity of Samothrace honeys and Manuka honey (4), with a significance level of $P< 0.05$.

In the determination of total flavonoids (Diagram 4.1.1.3), only a few samples (11, 1, 2, 3) exceeded the concentrations of the Manuka honey control that had a concentration of 1.32mM quercetin.

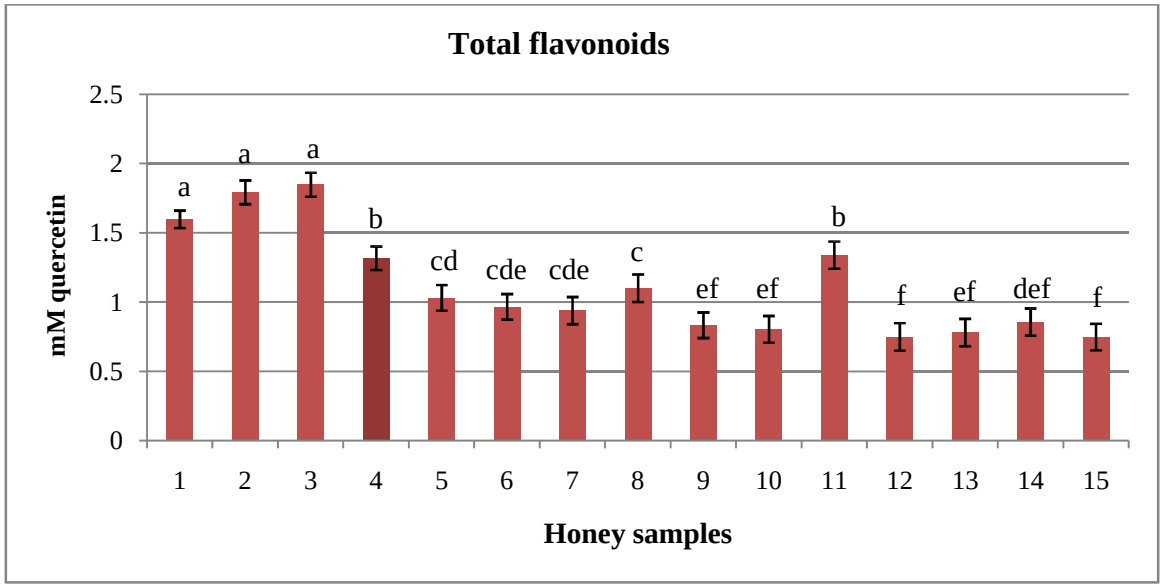


Diagram 4.1.1.3: Differences in total flavonoids of Samothrace honey and Manuka honey (4) with a significance level of P< 0.05.

4.1.2. Extractions of the *A. andrachne* plant

The determination of phenolic acids in the various parts of the plant showed that blossoms and fruits have very low concentrations, ripe leaves have higher concentrations than young ones, leaves have overall higher concentrations than blossoms and fruits and finally, extracts using β- cyclodextrin showed a significant difference reaching up to three times the concentration of phenolic acids for the same sample analyzed without β-cyclodextrin as well.

For example, the over-ripe leaves (R2) with the solvent with the best yield, which appeared to be the ratio of 80 ethanol: 20 water in a parallel experiment, showed a total phenolic concentration of 2533mgGAE/L, while using β-cyclodextrin they reached 7828mgGAE/L.

The following diagrams present the differences between the samples with a statistical significance level of P<0.05 based on the Tukey post hoc test. The lack of a common letter indicates statistically significant differences.

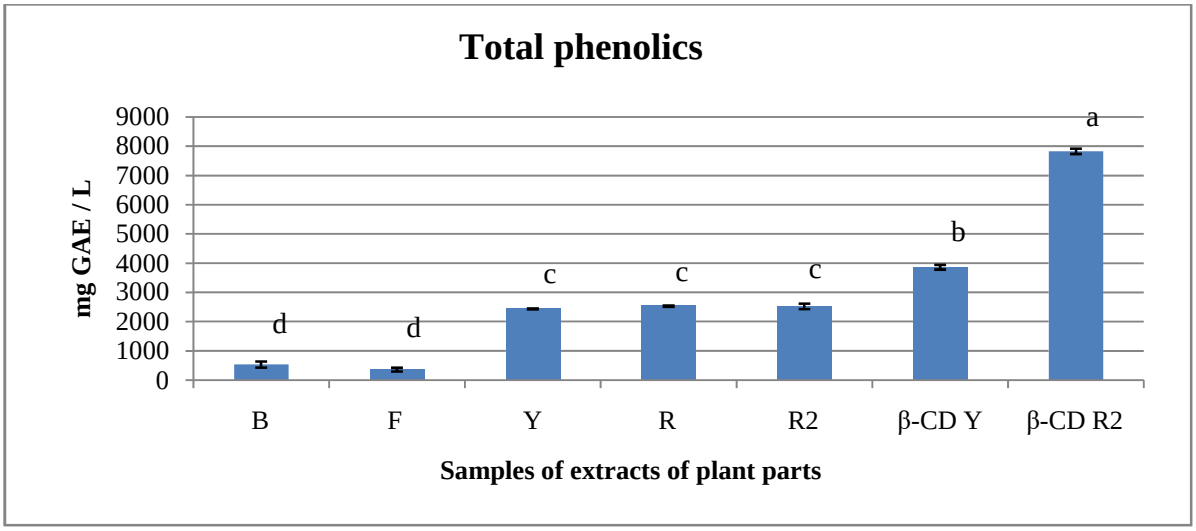


Diagram 4.1.2.1: Differences in total phenolic extracts of blossoms (B), fruits (F),young leaves (Y), ripe leaves (R), over-ripe leaves (R2), as well as young leaves with β-cyclodextrin (β-CD Y) and over-ripe leaves with β-cyclodextrin (β-CD R2) of the *A. andrachne* plant (from left to right), with a significance level of $P< 0.05$.

Concentrations interpreted as antioxidant capacity expressed in Trolox equivalents showed similar results, with blossoms and fruits having the lowest antioxidant capacity and leaves increasing antioxidant capacity during ripening. Here, too, a significant increase was observed with the use of β-cyclodextrin, since the values in both young and over-ripe leaves exceeded double, reaching about 42 and 54mMTRE, respectively.

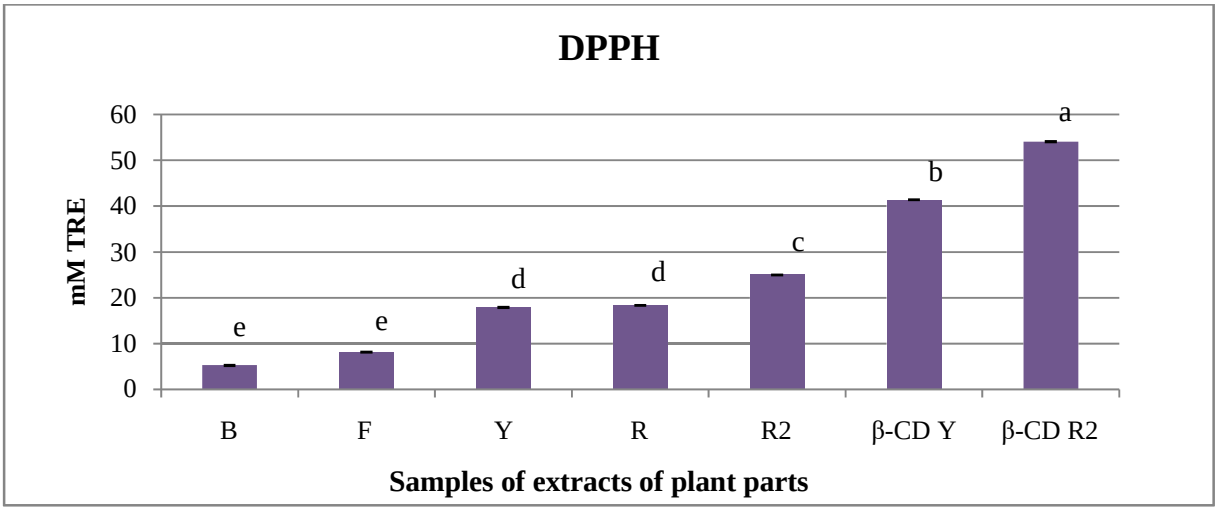


Diagram 4.1.2.2: Differences in antioxidant capacity of extracts of blossoms, fruits, young leaves, ripe leaves, over-ripe leaves, young leaves with β-cyclodextrin and over-ripe leaves with β-cyclodextrin of the *A. andrachne* plant (from left to right), with a significance level of $P< 0.05$.

In total flavonoids, while there appeared to be a slight decrease in the last stage of ripening, with over-ripe leaves having a lower concentration of flavonoids than ripe and young leaves, with the addition of β-cyclodextrin the young leaves reached almost twice their concentration (6.5mM quercetin) and the over-ripe ones exceeded the triple (8.6 mM quercetin).

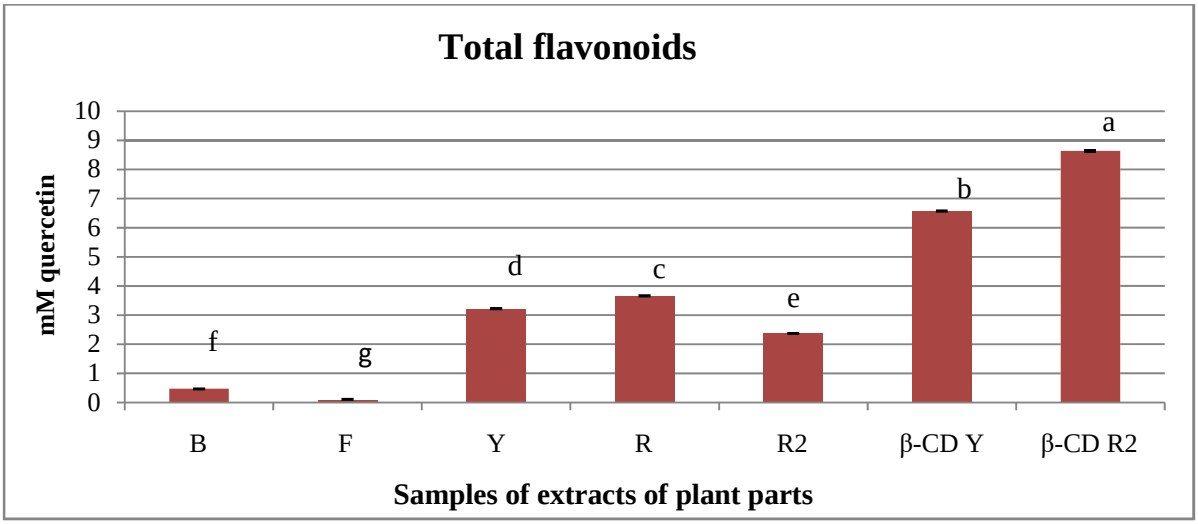


Diagram 4.1.2.3: Differences of total flavonoids of extracts of blossoms, fruits, young leaves, ripe leaves, over-ripe leaves, young leaves with β-cyclodextrin and over-ripe leaves with β-cyclodextrin of the *A. andrachne* plant (from left to right), with a significance level of $P< 0.05$

Various researches that have been conducted worldwide for the *A. andrachne* plant are also in agreement with the high values in the chemical characteristics of this plant. Tawaha et al. (2007) made comparisons of phenolic acids and antioxidants in various Jordanian plant species and observed that the highest concentrations were given by the plant *A. andrachne* followed by *Hypericum sp.*. More specifically, they recorded phenolic acid concentrations of 58.6mgGAE/g dry weight and antioxidant concentration of 731μmolTRE/g dry weight, in aqueous extracts of leaves of the plant.

Serce et al. (2010) focused on comparing the fruits of *A. unedo* and *A. andrachne* both morphologically and chemically. They concluded that *A. andrachne* has fruits richer in phenolic components and antioxidants with concentrations of 3343μgGAE/g fresh weight and 29.5μmolTRE/g fresh weight, respectively.

4.1.3. HPLC – Arbutin determination

With the High Performance Liquid Chromatography method, the concentrations of arbutin in the various plant parts were determined (Diagram 4.1.3.1). It was found that the

blossoms had the lowest concentration of all the parts of the plant and they were followed by the fruits which also had a very low concentration. The leaves showed arbutin concentrations which were multiple of these of the blossoms and fruits, with the young leaves surpassing the ripe ones, without statistically significant differences, however, and the over-ripe leaves with a concentration of almost 79μg/mL. This was the reason why apart from the over-ripe leaves, which showed the highest concentrations in all chemical analyses, the young ones were also selected for the creation of extracts with β-cyclodextrins.

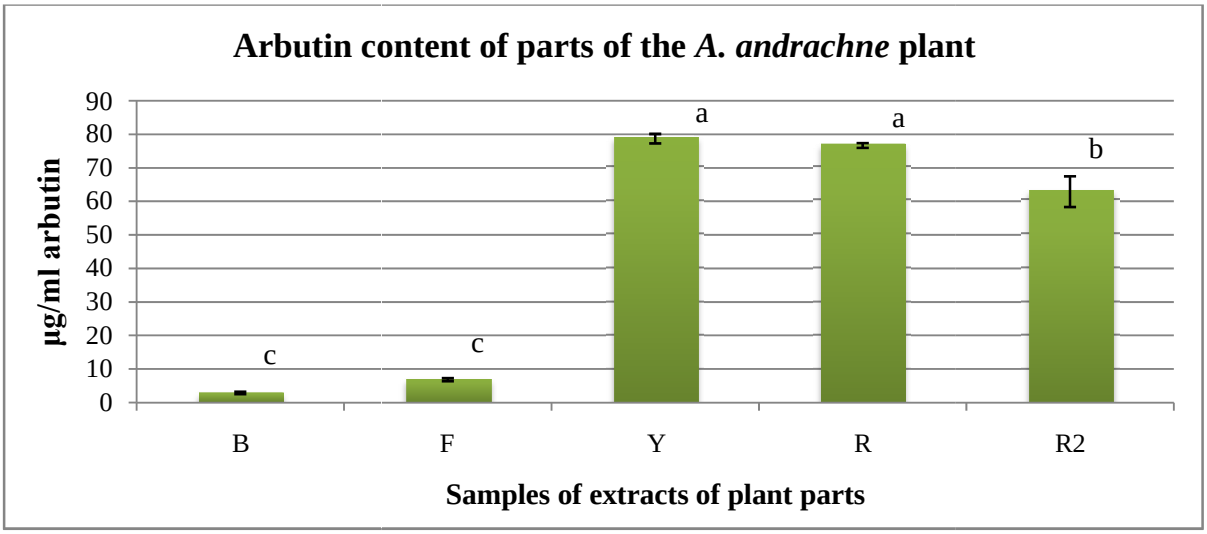


Diagram 4.1.3.1: Differences in arbutin content of extracts of blossoms, fruits, young leaves, ripe leaves and over-ripe leaves of the *A. andrachne* plant (from left to right), with a statistical significance level of $P< 0.05$.

Diagram 4.1.3.2 shows the chromatogram of the arbutin standard, with a concentration of 25μg/mL, in 6.95 minutes with a pressure of 1618psi. At the same pressure, the chromatogram of the extract of the young leaves of *A. andrachne* was also recorded, as shown in Diagram 4.1.3.3. As the pressure does not remain constant on the instrument throughout the duration of the analyses, it is clarified what pressure the minutes correspond to for each peak, because if the pressure rises on the instrument, which often happens when the analyses take place for many days, the minutes will be reduced. That is, at higher pressures from 1600 to almost 2000, arbutin showed peaks even earlier than 6.9 minutes.

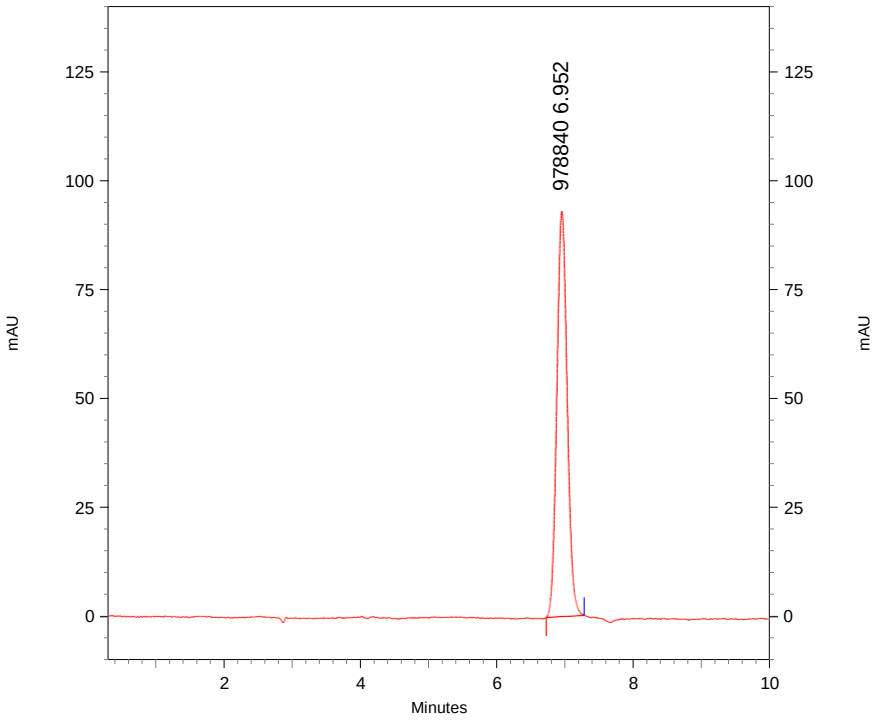


Diagram 4.1.3.2: Standard arbutin peak of known concentration of 25μg/mL.

Arbutin has also been found in many species of the Ericaceae family in various concentrations. Parejo et al. (2001) detected arbutin in leaves of *Arctostaphylos sp.* (Bearberry) at concentrations of 92μg/mg dry weight of the leaves with HPLC. In all the chromatograms of the parts of the plant there are other two, very specific, peaks that follow arbutin, much larger in comparison, which shows that these are substances with much higher concentrations (Diagram 4.1.3.3).

According to literature, there is a possibility that one of these substances is catechin, since according to Aljabari et al. (2014), in a study conducted for the *Arbutus andrachne* plant, a very similar HPLC protocol was used with column C18, at 280nm and solvent water: acetonitrile, and a catechin peak was obtained at 12.5 minutes and in fact at a concentration of 0.5mg / 100mg, ie 0.5% catechin content in the leaves of the plant. Therefore, since there are reports of such percentages in the same plant species, determined by a very similar protocol, it is very likely that some of the peaks shown in the chromatogram which were not determined to belong to catechin.

Also, Rosa et al. (2011) report that in the *Arbutus unedo* plant, which is a relative plant to *Arbutus andrachne*, there is a dominant phenolic, homogentisic acid, which accounts for 50-60% of the total phenolic acids of the plant and is even a phenolic marker. Therefore, due to affinity, there is a possibility that this phenol is also present in *Arbutus andrachne*, in some percentages and that it appears as one of the unidentified peaks of the chromatogram.

Finally, hydroquinone, which is the aglycone of the glycoside of arbutin, is very likely to be found in the chromatogram in a small percentage, and may have arisen naturally from some hydrolysis of glycoside.

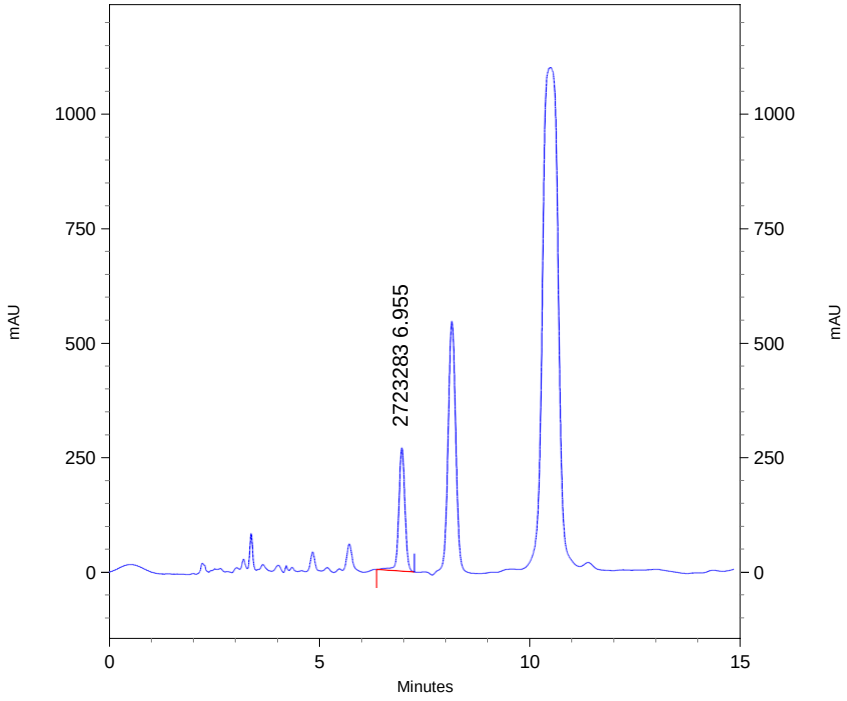


Diagram 4.1.3.3: Chromatogram of young leaf extract of the *A. andrachne* plant. The marked peak belongs to arbutin.

In Samothrace honeys, it was hypothesized that they may also contain quantities of arbutin, since a significant part of the nectar from which they came comes from the same plant. Diagram 4.1.3.4 shows a chromatogram of Samothrace honey which is characteristic and similar to all the honeys analyzed. Of course, there was no arbutin detected even in small quantities, and this is clearly visible in Diagram 4.1.3.5, where by placing the trace of the arbutin standard over the chromatogram of a honey, it appears that in that minute there is no peak corresponding to the arbutin.

The possible reasons why arbutin may not have been detected in honey can be many. Initially, very low content of arbutin was found in the blossoms of the *A. andrachne* plant, from which the bees receive nectar for the production of honey. Also, Samothrace honey comes from various endemic plants of the area, except *A. andrachne*, which most likely do not contain quantities of arbutin or at least quantities capable of changing the result. Finally, it is worth mentioning that arbutin is a phenolic glycoside chemically, and glycosides can be hydrolyzed. Therefore, the hydrolysis of glycoside by enzymes of the hypopharyngeal glands of bees, such as glucosidase (invertase), is not at all unlikely (Cianciosi et al. 2018).

Nevertheless, many other peaks corresponding to phenolic acids appear, since at the wavelength (280nm) observed, they absorb phenolic acids and corresponding solvents and columns are used for their analysis. The chromatograms of the honeys show that it is a rich honey in phenolic compounds, some of which are in not at all negligible concentrations.

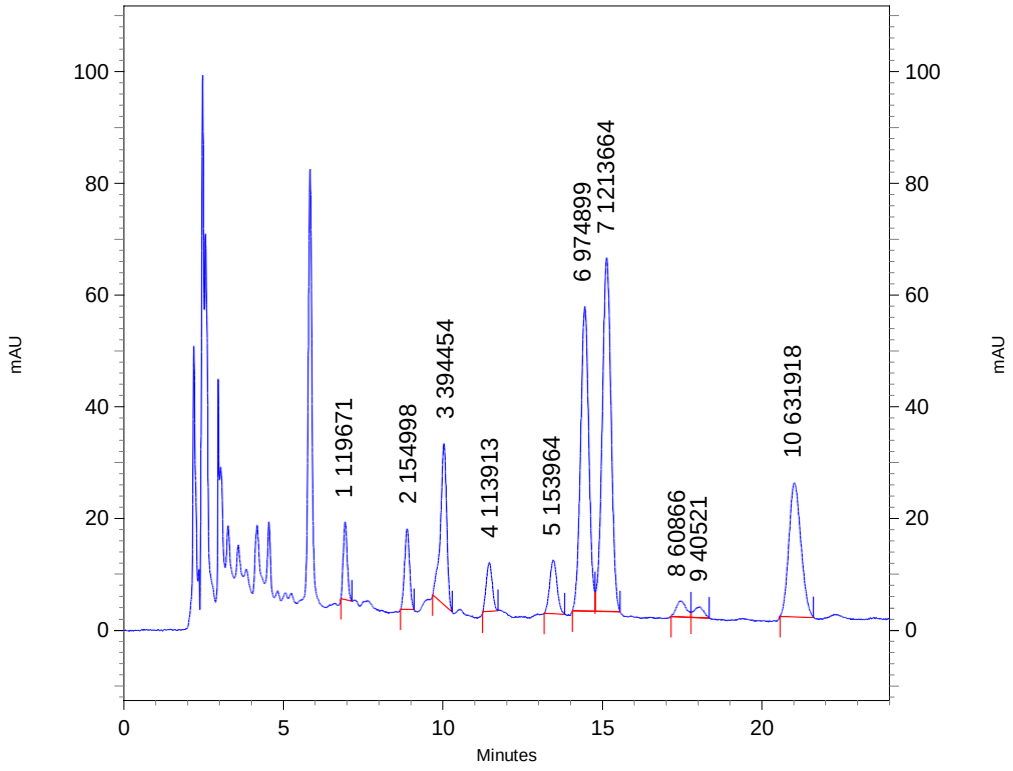


Diagram 4.1.3.4: Chromatogram of honey 1.

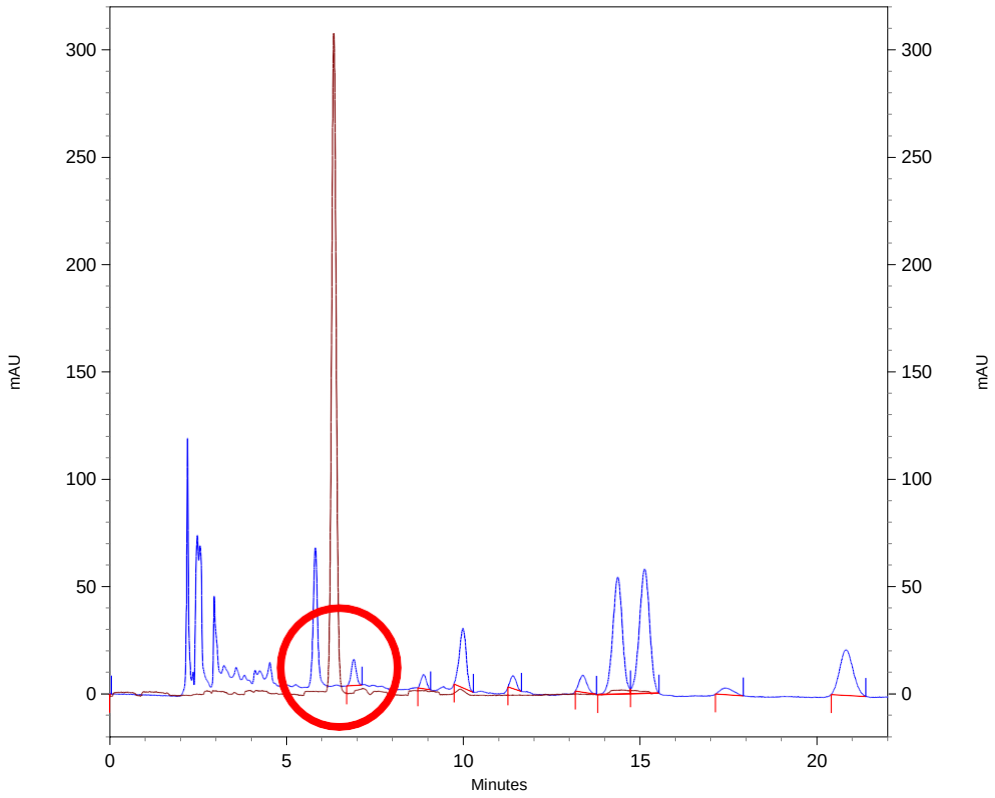


Diagram 4.1.3.5: Chromatogram of honey 2 (blue) and standard arbutin of known concentration 75μg/mL (brown).

4.2. Enrichments

4.2.1. Chemical analyses

Enriched honeys

Diagram 4.2.1.1 shows the concentrations of total phenolics in the enriched honeys. As it can be seen, β-cyclodextrin enrichment worked as expected, with sample 16, which had the highest percentage of β-cyclodextrin extract enrichment of over-ripe leaves, reaching a concentration of 1390mgGAE/L. There appears to be a significant difference, close to four times, from the original honey 1, which had a concentration of 345mgGAE/L. It should be noted here that the concentration of 880mgGAE/L (honey 12) was the highest that appeared in the determination of total phenolics of Samothrace honeys.

Sample 16 is followed by samples 20 (with 5% aqueous extract of over-ripe leaves) and samples 18 with a lower percentage of β-cyclodextrin extract of over-ripe leaves and 17, 19 with β-cyclodextrin extracts of young leaves. The controls showed lower concentrations than the original honey 1, as was reasonable, since they are a diluted version of honey, with water.

The statistically significant differences between the concentrations of the various percentages and solutions of enrichment for the chemical analyses of total phenolics, antioxidant capacity and total flavonoids, according to the Tukey test with a significance level of P< 0.05, are shown in the following Diagrams 4.2.1.1-3. The lack of a common letter indicates statistically significant differences.

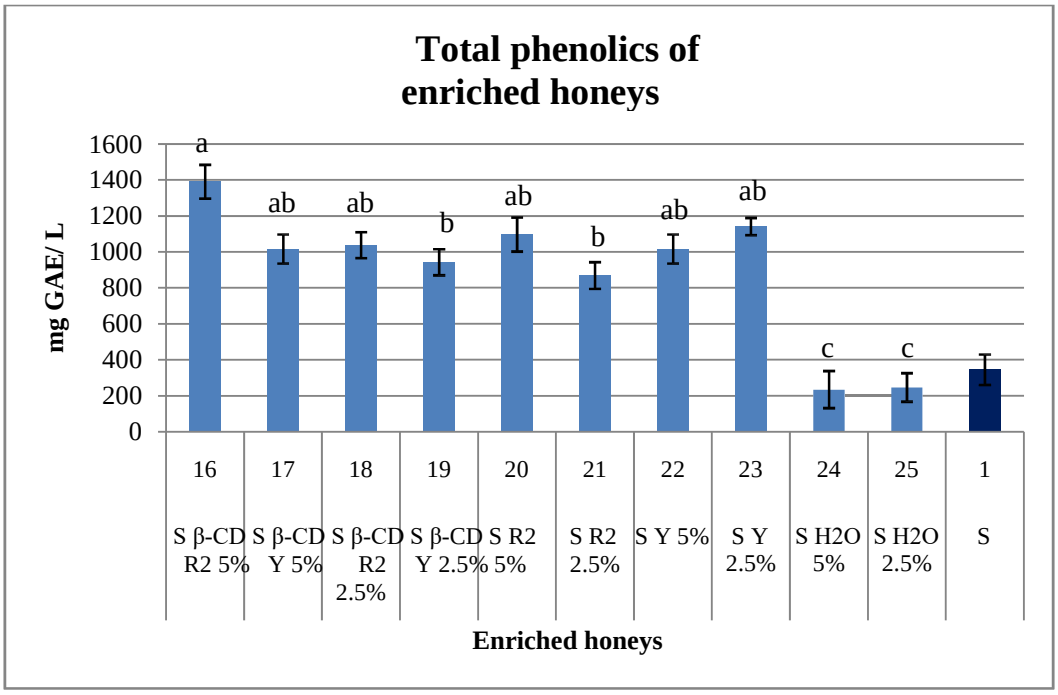


Diagram 4.2.1.1: Differences in total phenolics of enriched honeys (from left to right): honey 1 with 5% β-cyclodextrin extract of over-ripe leaves, honey 1 with 5% β-cyclodextrin extract of young leaves, honey 1 with 2.5% β-cyclodextrin extract of over-ripe leaves, honey1 with 2.5% β-cyclodextrin extract of young leaves, honey 1 with 5% aqueous extract of over-ripe leaves, honey 1 with 2.5% aqueous extract of over-ripe leaves, honey 1 with 5% aqueous extract of young leaves, honey 1 with 2.5% aqueous extract of young leaves, honey 1 with 5% water, honey 1 to 2.5% water, honey 1, with a significance level of P< 0.05.

The antioxidant capacity of enriched honeys, as shown in Diagram 4.2.1.2, showed a significant increase, approaching three times the initial concentration of honey 1, with the addition of 5% β-cyclodextrin extract of over-ripe leaves (sample 16), reaching a concentration of 5.8mMTRE. This was followed by the aqueous solutions of the over-ripe leaves with a higher concentration of 4.9mMTRE at the highest enrichment rate.

In young leaf extracts, it was observed that aqueous extracts were superior in antioxidant concentrations than those with the addition of β-cyclodextrin, but there were no statistically significant differences.

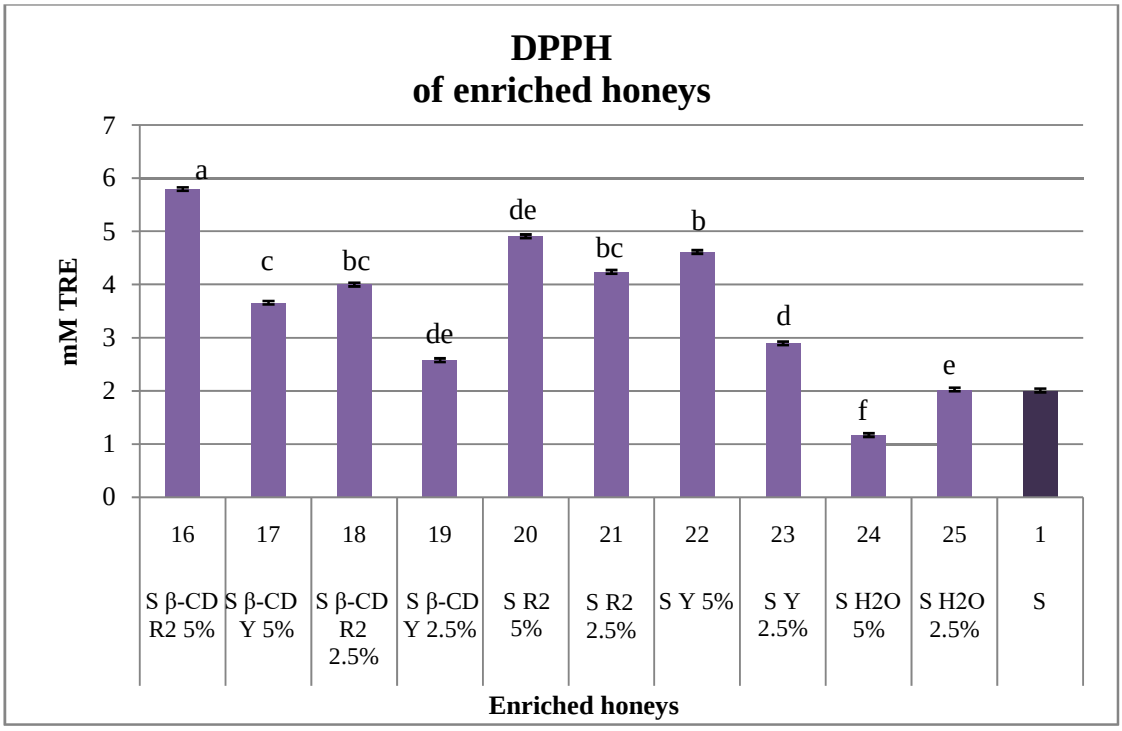


Diagram 4.2.1.2: Differences in antioxidant capacity of enriched honeys (left to right): honey 1 with 5% β-cyclodextrin extract of over-ripe leaves, honey 1 with 5% β-cyclodextrin extract of young leaves, honey 1 with 2.5% β-cyclodextrin extract of over-ripe leaves, honey1 with 2.5% β-cyclodextrin extract of young leaves, honey 1 with 5% aqueous extract of over-ripe leaves, honey 1 with 2.5% aqueous extract of over-ripe leaves, honey 1 with 5% aqueous extract of over-ripe leaves, honey 1 with 5% aqueous extract of young leaves, honey 1 with 2.5% aqueous extract of young leaves, honey 1 with 5% water, honey 1 to 2.5% water, honey 1, with a significance level of $P< 0.05$.

While in the determination of total phenolics and antioxidant activity, the addition of β-cyclodextrin significantly increased the concentrations in relation to the concentrations shown in the samples with the addition of aqueous extract, in total flavonoids nothing similar was observed. As shown in the diagram 4.3.1.3, the concentrations of aqueous extracts exceed the concentrations of extracts with the addition of β-cyclodextrin. For example, sample 20 compared to sample 16 showed concentrations of 2.65mM quercetin and 2.4mM quercetin respectively, the first being enrichment with 5% aqueous solution of over-ripe leaves and the second one corresponding enrichment with addition of β-cyclodextrin. The same was observed in samples 22 and 17 with young leaf extracts with concentrations of 2.54mM quercetin and 2.22mM quercetin, respectively.

However, the 1.6mM quercetin concentration of non-enriched honey 1 increased with enrichment. As a possible explanation to the above, it could be mentioned that β-cyclodextrins did not work as expected, perhaps because they were not suitable for the specific flavonoid molecules. Cyclodextrins α-, β-, γ-, have different sizes and different molecules can be enclosed accordingly, so α- or γ- cyclodextrin may be needed for greater yield if the specific flavonoid molecules were smaller or larger, respectively.

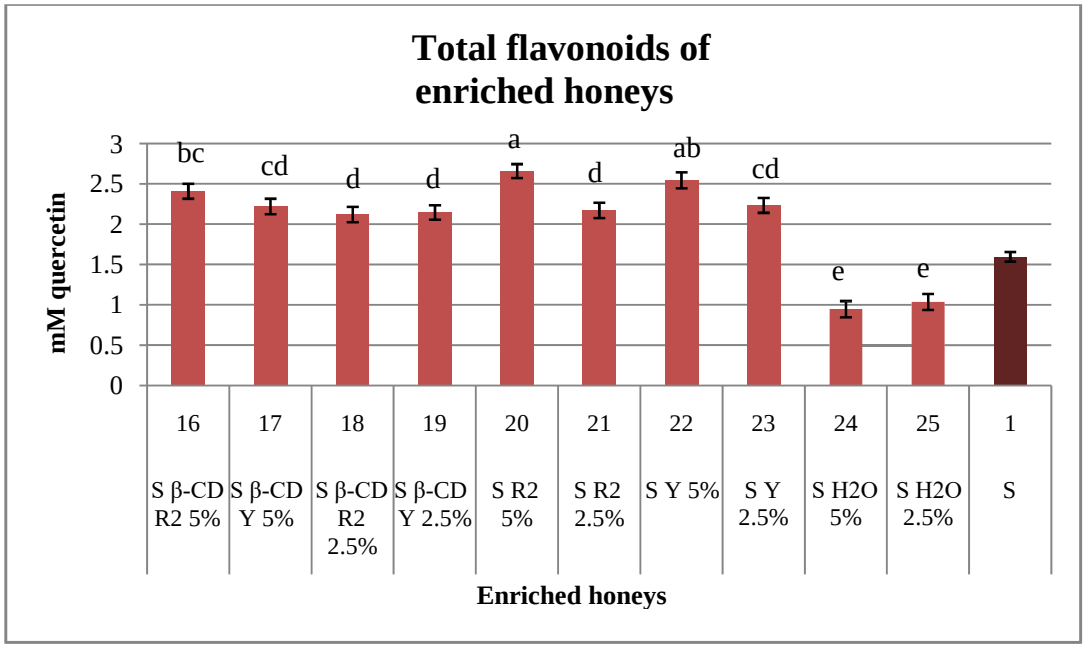


Diagram 4.2.1.3: Total flavonoid differences of enriched honeys (from left to right): honey 1 with 5% β-cyclodextrin extract of over-ripe leaves, honey 1 with 5% β-cyclodextrin

extract of young leaves, honey 1 with 2.5% β-cyclodextrin extract of over-ripe leaves, honey1 with 2.5% β-cyclodextrin extract of young leaves, honey 1 with 5% aqueous extract of over-ripe leaves, honey 1 with 2.5% aqueous extract of over-ripe leaves, honey 1 with 5% aqueous extract of young leaves, honey 1 with 2.5% aqueous extract of young leaves, honey 1 with 5% water, honey 1 to 2.5% water, honey 1, with a significance level of $P< 0.05$.

More generally in literature, honey enrichments, although not as frequent, seem to work well, since the new enriched honey is significantly fortified in polyphenols, which is mainly what the various researches are interested in determining. For example, in the research of Tumbas et al. (2012) in which they enriched acacia honey with prunes, the highest percentage of enrichment (40%) reached a concentration of 41.64mgGAE/100g compared to the control 16.18mgGAE/100g. In the determination of flavonoids, the difference was even greater with the control showing a concentration of 2.65mgRE/100g and the highest concentration of enrichment reaching 30.86mgRE/100g.

Similar results were obtained by Socha et al. (2015), who chose to enrich honey with beekeeping products. While control honey, a simple blossom honey of the region, had a phenolic concentration of 36mgGAE/100g, with the addition of royal jelly (0.5-0.8%) it had a phenolic concentration of 40mgGAE/100g, with the addition of pollen (5-10%) 84mgGAE/100g, with the addition of propolis (0.5-1%) 114mgGAE/100g and with the addition of "beebread" 180mgGAE/100g.

Also, in the experiment of Stajner et al. (2014) the maximum amount of enrichment (10%) of acacia honey with *Rosa spp.* fruit showed a phenolic concentration of 154.61mgGAE/100g while the control ranged from 16 to 17mgGAE/100g.

As observed, depending on the concentration of phenolics, for example, that the enrichment substance has, the enriched product will fluctuate accordingly. Since the extract of the *A. andrachne* plant is so rich in phenolic compounds and antioxidants, it is almost expected that there will be very large differences compared to the control, since from 345mgGAE/L, with the enrichment they reached 1096mgGAE/L and 1390mgGAE/L with simple aqueous extract and β-cyclodextrin extract, respectively.

4.2.2 HPLC-Arbutin determination

In the enriched honeys, an attempt was made to determine the arbutin, which was naturally present in the plant extract. While in all enriched honeys a low flattened peak was observed at 6.25 minutes with a pressure of 1740psi, as noted by an arrow in the chromatogram of Figure 4.3.3.1, it was not possible to quantify this small peak, since it was much lower than the lower limit of the reference curve. However, since Samothrace honeys, as mentioned above, did not show any arbutin peak, even this small peak corresponds to 5% of the extract added, which proves in a way the enrichment with the extract.

This can be confirmed by the chromatogram of Figure 4.3.3.2 showing the honey sample in blue, simultaneously with the trace of the standard arbutin in brown, whose peaks coincide. It is therefore the same substance. Also, from the chromatograms of the enriched honeys it is confirmed that the young leaves have higher amounts of arbutin, since larger peaks emerged in the honeys enriched with extracts of young leaves.

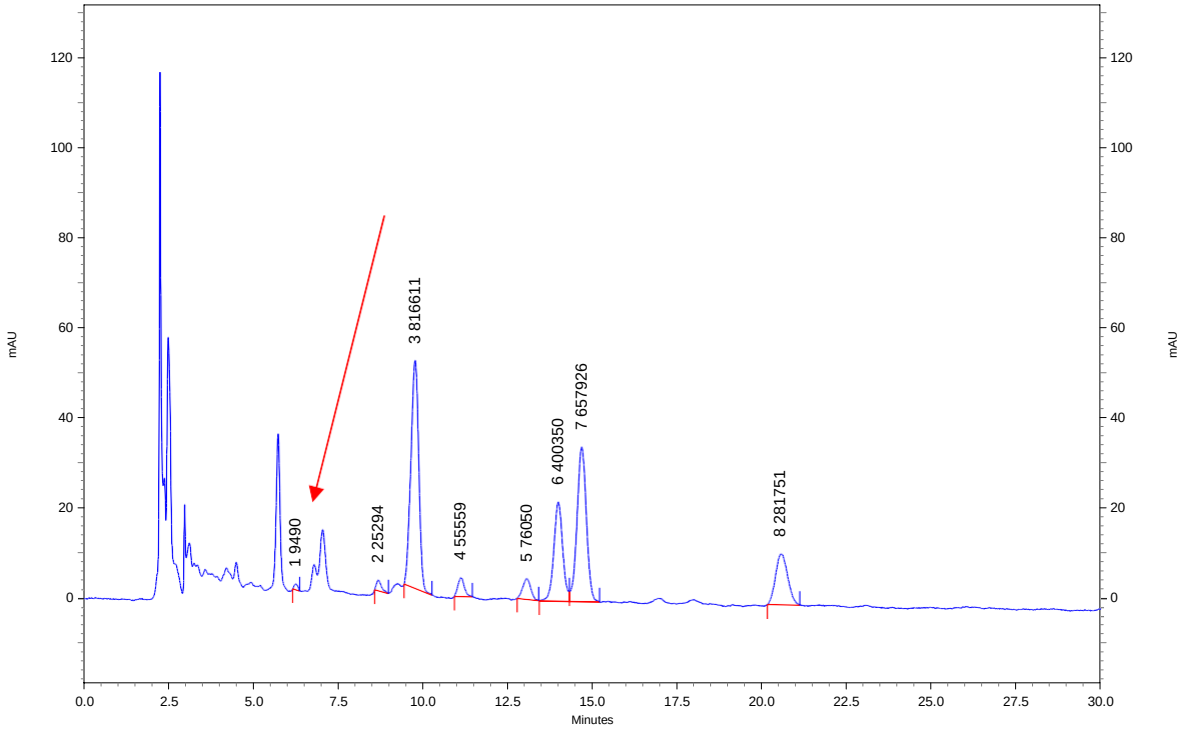


Figure 4.2.2.1: Chromatogram of enriched honey with 5% over-ripe leaf extract with β-CD

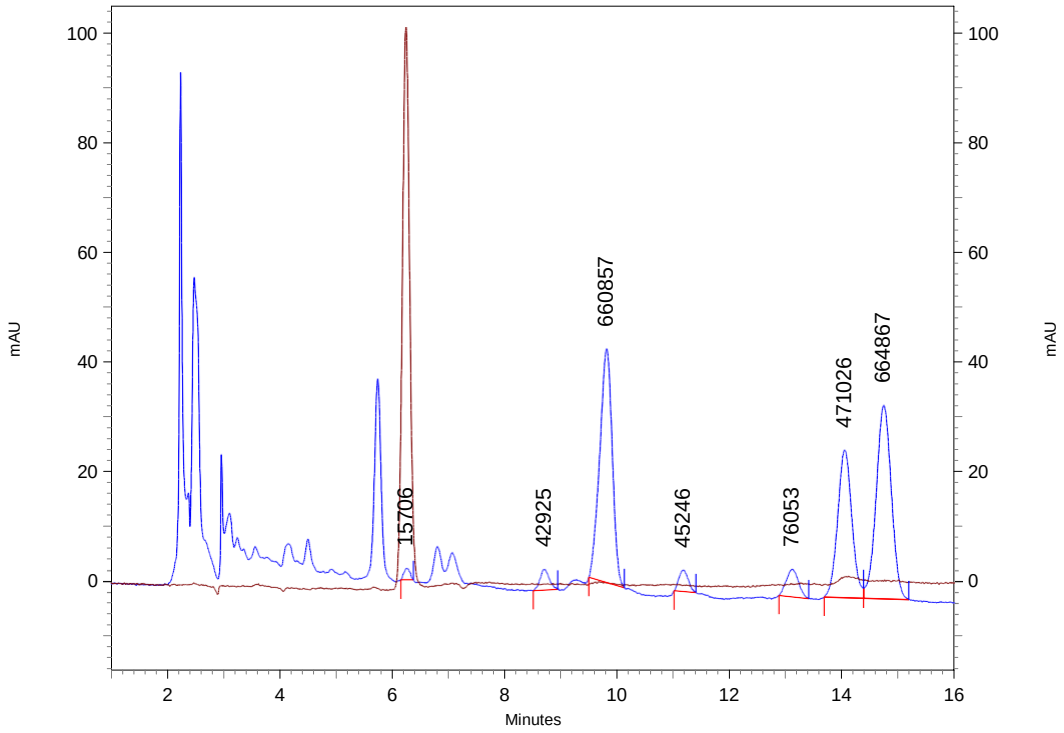


Figure 4.2.2.2: Chromatogram of enriched honey with 5% extract of young leaves with β-CD (blue) and standard arbutin (brown).

4.3. Microbiological tests

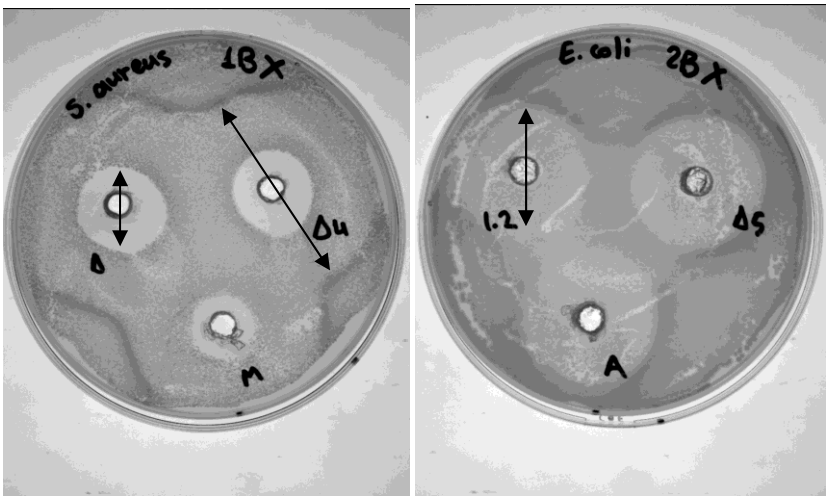
4.3.1. Samothrace Honeys

In all repetitions, two types of zones of inhibition (Figure 4.3.1-2) were observed, clear zones and cloudy zones.

Each pathogen showed a different sensitivity, in the presence of honey, as shown in Table 4.3.1. It was the bacterium *Staphylococcus aureus* that showed the greatest sensitivity to the presence of honey (thick and diluted 50%), since clear inhibition zones were observed around the well. The indicative figures (Figure 4.3.1.1) show the clear zones with smaller diameters and the cloudy zones with larger diameters, in thick honey.

The cloudy zones (Figure 4.3.1.1-2) were observed in all honey samples and controls (artificial honey A (sugars + extract) & artificial honey B (sugars + water)) and could be attributed to the difference in osmotic potential, due to very high sugar concentrations in the samples. For *Staphylococcus aureus* in the presence of thick honey samples and controls, cloudy

zones observed ranged in diameter 4.35-4.65cm, and in the presence of 50% diluted samples the diameter recorded was 3.8-4cm.



Figures 4.3.1.1-2: Clear and cloudy inhibition zones of pathogens *S. aureus* and *E.coli* in the presence of thick Samothrace honeys (S, S4, S5, 1.2), Manuka honey (M) and artificial honey A.

The bacterium *Escherichia coli* appeared to be more resistant to the presence of honey and osmotic pressure. Primarily cloudy zones were observed (Figure 4.3.1.2) in the presence of all honeys except honeys 8-14, where it also showed clear zones in both thick and diluted honeys, although smaller in diameter (0.9-1.2cm and 0.5-0.62cm respectively) than the zones against *Staphylococcus aureus*. The cloudy zones for the thick samples were observed to be in the range of 2.9-3.25cm and for the diluted (50%) samples 1.8-2.45cm.

The bacterium *Listeria monocytogenes* appeared to be the most resistant of all in the presence of honey, since it only showed cloudy zones, very small in diameter (0.95-1.35cm), in only a few honeys and only in tests with thick honey.

In the presence of artificial honey A and B, inhibition zones were also observed, which could be attributed to the difference in osmotic potential. The cloudy zones in the presence of artificial honey B were similar to the cloudy zones of Samothrace honey.

The above results are in line with most research results that studied antibacterial activity of honeys, identifying differences of zones of inhibition. Voidarou et al. (2011) observed that *Staphylococcus aureus* and *Escherichia coli* showed inhibitions regardless of honey origin, with the first being the most sensitive. They also noticed that the largest inhibitions were created by coniferous and thyme honey.

Huttunen et al. (2012) also agree, since *Staphylococcus aureus* had zones of inhibition in all honeys tested except in the honey from the *Rubus chamaemorus* plant. Similarly, *Streptococcus pneumoniae* was sensitive to all honeys, followed by *Streptococcus pyogenes*, which was inhibited in all honey concentrations (20-60%).

Avocado and blueberry honey caused the largest inhibition zones in *Staphylococcus aureus* of all honey tested, according to Taormina et al. 2001, and they also conclude that the most susceptible bacterium of the six studied was *Staphylococcus aureus* and the most resistant one, the least affected of all, was *Bacillus cereus*. Brazil honeys, on the other hand, tried by Bueno- Costa et al. 2016 against four pathogens, showed very good inhibition in both *Staphylococcus aureus* and *Bacillus cereus*.

In China, Deng et al. (2018) tested buckwheat honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with a similar method to the present experiment, and observed that it had a similar inhibition to Manuka honey. Same observations were recorded by Anthimidou & Mossialos (2012) and Stagos et al. (2018), the first ones with Greek and Cypriot honeys and the second one with Olympus honeys, concluding that the honeys had very good inhibitions, similar to Manuka and some honeys even exceeded it in inhibition, especially in the case of *Staphylococcus aureus* which was again the most sensitive bacterium.

Table 4.3.1 below summarizes the results of the inhibition activities that the honey samples and control-artificial honey B sample appeared to have against the three microorganisms, except for the activities due to the difference in osmotic potential. As more complete, the results of the thick honeys were used for the statistical analysis.

Table 4.3.1.1: Antibacterial activity of honey samples (Samothrace, Manuka, and artificial honey), thick and diluted with PBS, against three pathogenic bacteria, as it emerged from the examination using the Agar Well Diffusion Assay.

		Staphylococcus aureus		***Escherichia coli***		***Listeria monocytogenes***	
		Inhibition (zone diameter in cm)*					
Samples		**Thick**	**50%**	**Thick**	**50%**	**Thick**	**50%**
S	1	2 de **	1.45	- d	-	(1.35) bcd ***	-
S4	**2**	2 de	1.6	- d	-	(1.4) bc	-
S5	**3**	1.95 e	1.55	- d	-	(1.7) a	-
M	**4**	1.55 f	1.3	- d	-	(1.15) def	-
1.2	**5**	1.3 f	0.9	- d	-	- g	-
1.3	**6**	1.35 f	1.35	- d	-	(0.95) f	-
3.3	**7**	1.4 f	1.05	- d	-	(0.95) f	-
9.1	**8**	2.3 ab	1.75	1.1 ab	0.62	(1.17) de	-
9.2	**9**	2.4 abc	1.7	1.1 ab	0.6	(1.2) cde	-
9.3	**10**	2.4 ab	1.65	1.1 ab	0.6	(1.13) ef	-
9.4	**11**	2.46 a	1.8	1.2 a	0.6	(1.16) cde	-
9.5	**12**	2.23 cde	1.6	0.9 c	0.55	(1.1) ef	-
9.6	**13**	2.16 bcde	1.58	1.17 ab	0.65	(1.23) cde	-
9.7	**14**	2.3 abc	1.67	1.07 ab	0.5	(1.17) cde	-
9.8	**15**	2.2 bcd	1.6	1.03 b	0.5	(1.2) cde	-
B	**27**	- g	-	- d	-	- g	-

*Averages (of three repetitions) in the same column with a different letter, differ statistically at a significant level, with a significance level of $P < 0.05$.

**The diameter is recorded along with the diameter from the well (0.5cm).

***Values in brackets indicate cloudy zones of inhibition.

The catalase test showed that the antibacterial activity of the honeys is due to the presence of a large amount of hydrogen peroxide, since after the addition of catalase to the honey solution, and after incubation, no inhibition zones were formed, meaning that the microorganism *S. aureus* developed normally. In Figure
4.3.1.3 and in Table 4.3.3 the activity of catalase is shown in a a distinctive way. In most honeys, the presence of hydrogen peroxide is almost exclusively responsible for the antibacterial activity.

In the research of Taormina et al. (2001), antibacterial activity was tested on six pathogenic bacteria: *Escherichia coli* O157:H7, *Salmonela typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*. In the catalase test, the bacteria *Shigella sonnei*, *Listeria monocytogenes* and *Staphylococcus aureus* showed greatly reduced inhibition in the presence of catalase, which implies the antibacterial activity of hydrogen peroxide.

Snow & Manley-Harris (2004) published that the activity of Manuka honey against *Staphylococcus aureus* is not due to the presence of hydrogen peroxide, since they did not observe differences in the inhibition zones in the presence of catalase.

Table 4.3.1.2: Testing of antibacterial activity of samples by adding catalase against *S. aureus.*

		50% PBS	50% catalase
Samples		Inhibition (cm diameter)	Inhibition (cm diameter)
S	**1**	1.45	-
S4	**2**	1.55	-
S5	**3**	1.45	-
1.2	**5**	1	-
1.3	**6**	1.3	-
3.3	**7**	1	-
9.1	**8**	1.8	-
9.2	**9**	1.7	-
9.3	**10**	1.75	-
9.4	**11**	2	-
9.5	**12**	1.7	-
9.6	**13**	1.6	-
9.7	**14**	1.7	-
9.8	**15**	1.7	-

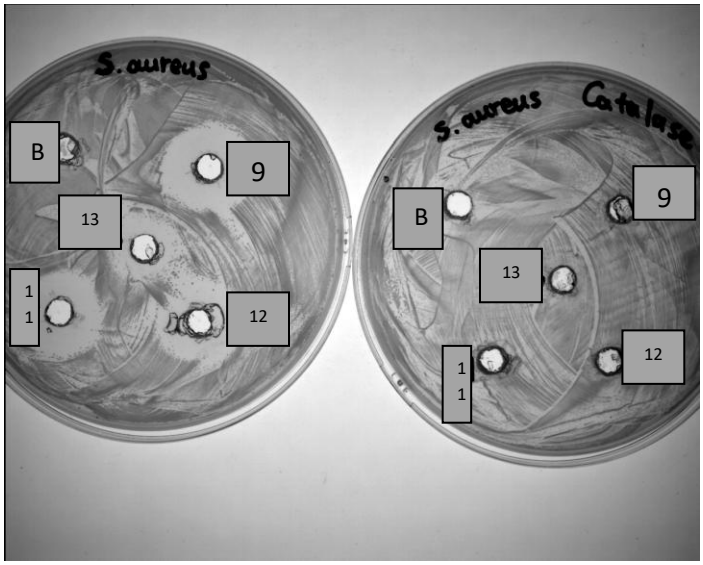


Figure 4.3.1.3: Inhibition zones of the pathogen *S. aureus* in the presence of honey solutions in 50% concentration, without catalase (left) and with catalase (right).

4.3.2. Enriched honeys

For the study of the antibacterial activity of honey enriched with extract of the *A. andrachne* plant, honey 1 was selected to be enriched.

In relation to the results of the thick and diluted by 50% Samothrace honeys, the samples of honey enriched with different plant extracts (Samples 16-25) showed corresponding, slightly increased inhibitions at the highest rates of enrichment in the bacterium *S. aureus* (Table 4.3.2.1).

The inhibition zones in *S. aureus* ranged from 1.65 to 2.35cm for thick samples and 1.1-1.7cm for 50% diluted samples.

Regarding the bacterium *E. coli*, inhibition zones were observed in samples 16, 17 and 20.

Finally, in the bacterium *L. monocytogenes*, inhibition zones (0.55-0.95cm) were observed only in thick samples, but smaller than those in Samothrace honeys (Samples 1-15). The clear zones observed in the presence of artificial honey A were statistically significantly smaller than the clear zones of Samothrace honeys.

In other researches in which honey enrichments were attempted, such as in Tumbas et al. (2012) in which they enriched honey with prunes, in Socha et al. (2015) in which they tried enrichment with various beekeeping products and in Stajner et al. (2014) in which they enriched honey with *Rosa spp.*fruits, they focused on the determination of total phenolic compounds and antioxidant capacity, as well as on the comparison of the results before and after the enrichment. In none of the above bibliographic references for honey enrichment was an antibacterial activity test of enriched honeys performed.

Table 4.3.2.1: Antibacterial activity of honey samples (enriched with *A. andrachne* plant extracts), thick and diluted with PBS, against three pathogenic bacteria, as it emerged from the examination using the Agar Well Diffusion Assay.

		Staphylococcus aureus		***Escherichia coli***		***Listeria monocytogenes***	
		Inhibition (zone diameter in cm)*					
Samples		**Thick**	**50%**	**Thick**	**50%**	**Thick**	**50%**
S β-CD R2 5%	**16**	2.35 a **	1.7 b	0.85 c	-	0.95 b	-
S β-CD Y 5%	**17**	2.25 a	1.6 bc	0.55 d	-	0.6 cd	-
S β-CD R2 2.5%	**18**	1.65 cd	1.1 e	- e	-	0.55 d	-
S β-CD Y 2.5%	**19**	2.15 a	1.5 bc	- e	-	0.55 d	-
S R2 5%	**20**	2.2 a	1.55 bc	0.95 b	-	0.9 bc	-
S R2 2.5%	**21**	2.1 ab	1.1 bc	- e	-	0.75 bcd	-
S Y 5%	**22**	2.15 a	1.5 bc	- e	-	0.85 bcd	-
S Y 2.5%	**23**	2 abc	1.45 cd	- e	-	0.55 d	-
S H2O 5%	**24**	1.7 cd	1.15 de	- e	-	0.65 bcd	-
S H2O 2.5%	**25**	1.75 bcd	1.1 de	- e	-	0.55 d	-
A	**26**	1.4 d	0.95 a	3 a	2.55	1.55 a	-

*Averages (of three repetitions) in the same column with a different letter, differ statistically at a significant level, with a significance level of $P < 0.05$.

**The diameter is recorded along with the diameter from the well (0.5cm).

Chapter 5 – Conclusions

Summarizing, the enrichment of Samothrace honey worked particularly well in terms of the chemical characteristics of the final product, since it resulted in a honey with almost triple antioxidant activity and almost quadruple total phenolic content.

More specifically, Samothrace honeys appeared to be quite strong honeys, both in terms of antioxidant activity and polyphenol concentration and in terms of antibacterial activity. The antioxidant activity of Samothrace honey calculated in Trolox equivalents reached 2.87mMTRE compared to Manuka honey which had a concentration of 1.25mMTRE. Regarding the total phenolic content, an average of around 590mgGAE/L was recorded, with the Manuka honey control reaching a concentration of about 349mgGAE/L. In the determination of total flavonoids, however, only 4 Samothrace honey samples exceeded the concentration of 1.32mMQE belonging to the Manuka honey control.

In microbiological tests, Samothrace honeys showed clear and cloudy zones of inhibition against pathogens. More specifically, *Staphylococcus aureus* appeared to be the most susceptible one, since clear zones of inhibition with a significant diameter were formed, even in the presence of lower concentrations of honey. The bacterium *Escherichia coli* was slightly more resistant to the presence of honey. However, small in diameter zones of inhibition were observed in the presence of several samples. Finally, only a few honeys were able to inhibit the bacterium *Listeria monocytogenes* and only in tests with thick honey. It is important to mention that in almost all cases, Samothrace honeys surpassed Manuka honey in inhibition diameter. This inhibition ability of honey against pathogens appeared to be mainly due to the high concentration of hydrogen peroxide, as shown by the catalase test, and then to the high concentration of sugars and therefore to the difference in osmotic potential.

The *Arbutus andrachne* plant was verified to be a plant with very high concentrations of polyphenols as well as antioxidants. The young, ripe and over-ripe leaves of the plant had the highest concentrations in all chemical analyses.

It was also observed that with the extraction of the same plant parts with β-cyclodextrin, the concentrations of total phenolics reached the triple, the antioxidant activity exceeded twice and the concentration of total flavonoids moved correspondingly.

Arbutin was found to be present at a higher percentage in the young leaves of the plant, using High Performance Liquid Chromatography. Samothrace honey did not appear to contain arbutin, while in the enriched honey with extract from the *Arbutus andrachne* plant, a very low, flattened, non-quantifiable peak of arbutin appeared.

Finally, regarding the enrichment of honeys, there was a significant increase in the concentrations of chemical characteristics. The concentration of total phenolics, from 345mgGAE/L for honey to be enriched, reached 1390mgGAE/L with the addition of 5% β-cyclodextrin extract of over-ripe leaves, i.e. it quadrupled. The differences in the antioxidant activity of honeys moved correspondingly. However, in the concentrations of total flavonoids it was not observed something similar. In fact, enriched honey samples with aqueous extracts showed higher concentrations than the honeys enriched with β-cyclodextrin extracts. In microbiological tests, samples of enriched honey with different percentages of extracts from the *A. andrachne* plant did not affect the antibacterial activity of the original samples (similar responses were observed with thick, unprocessed honeys) of Samothrace, against pathogenic bacteria. Artificial honey (sample A) with over-ripe leaf extract showed inhibition activity against *S. aureus*.

In conclusion, this proposal of enriched honey is a promising idea for the industry, as it includes many advantages. Fortifying honey with natural ingredients is feasible, simple and at a low cost, with the additional benefit of the consumer's intake of a plethora of antioxidants and polyphenols, for which there is strong evidence that they work preventively and therapeutically in favour of human health. Of course, it is necessary to conduct additional research perhaps on the improvement of the enrichment process and the inhibition of microorganisms. The comprehensive research on this product also requires the control of the organoleptic characteristics in order to investigate whether and to what extent it is acceptable by the consumers.

Chapter 6 –References

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It is hereby certified that, to the best of the signer's knowledge and beliefs, this is a true and accurate translation of the attached Greek document into English.

The Translator
Chrysoulaki Chrysoula
10 I. Kazika str.-55132
Thessaloniki, Greece
PAT Reg. No.: 808

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