# Compositional profile of food supplements for honeybees

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*Dissertation submitted to Escola Superior Agrária de Bragança to obtain the Degree of Master in FOOD QUALITY AND SAFETY under the scope of the double diploma with Institut Supérieur Privé Polytechnique – Université Libre de Tunis*

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Bragança

2019

## ACKNOWLEDGEMENTS

Firstly, I am grateful to my supervisor Soraia Falcão. She was always willing to help me, give me advice, support and courage and guide me in my research. She makes me fascinated with the world of honeybees. Working with her has been a great honor, and I will always consider myself lucky that I had her as an advisor and supporter.

I am also grateful to my co-supervisors Miguel Vilas Boas and Faten Essid, who were always very willing to help me when I needed them, adding a lot to my work.

I am also very thankful to Elmehdi Louraouine and Andréa Tomas, for their kindness, they were always willing to help and give advice in the laboratory.

My warmest thanks goes to Mr Yassine for the opportunity I had to study in IPB, acquiring more knowledge and having a nice experience.

Working in the CIMO has been a wonderful experience due to the great people in this laboratory, I am thankful to all for making this research possible and for the great atmosphere you created;

Of course, none of this could happen without the support and the unconditional love of my adorable family. I am grateful for my beloved mother Najoua, for giving me hope and filling me with strength in every step of this work, my great father Bassem for his trust, encouragement and advices in all the steps of my life, my two brothers Firas and Adam for their support and for believing in me. Thank you for being there for me, thank you for helping me achieve my dreams!

The author thanks to Programa Apícola Nacional (2017-2019) for the funding to the Apiscibus and to Fundação para a Ciência e Tecnologia (FCT) and FEDER inside the Program PT2020 for the finantial support to CIMO (UID/AGR/00690/2019).

## ABSTRACT

Honeybees (*Apis mellifera* L.) are the main pollinating agents for numerous plants and fruit trees and, hence, play a key role in agriculture and more generally in the maintenance of ecological biodiversity.

Like the most of organisms, honeybees need a diverse diet consisting of minerals, carbohydrates (sugars), fats, and amino acids (proteins) to survive and reproduce. An adult honey bees carbohydrate requirement is satisfied by the nectar produced in flowers and also occasionally from extra floral nectarines or honeydew secreted by plant-feeding insects, while, flower pollen is the main source of amino acids, protein building blocks, largely used to feed developing larvae and young bees to provide structural elements of muscles, glands and other tissues.

Bee-population declines are linked to nutritional shortages caused by land-use intensification, which reduces diversity and abundance of plant species. Together with the deficiency caused by adverse climatic changes and with the need to reduce colony mortality and particularly to ensure good nutritional/health status of bees in specific production moments, artificial supplementation of honeybee colonies became a major issue in beekeeping, and now is a common and growing practice within Portuguese beekeepers. This practice, in association with the reduced regulation in this area is leading to the proliferation of commercial products based on carbohydrates, protein and other substances of diverse origins and compositions. The impact of these products on hives may enable the beekeeper to remedy colony imbalances resulting from adverse or beekeeping conditions, but also may pose risks to the bee's health and the bee products quality, depending on the used raw materials and the presence of harmful substances.

The present work, inserted within the project ApisCibus - Artificial food for honeybees: quality survey, digestibility and performance on the bee hive, will have as main objective to evaluate the quality of commercial honeybee artificial supplementation through composition analysis of commercial supplements. The

quality parameters evaluated are: minerals quantified through atomic absorption spectroscopy, fatty acids analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and amino acids which are analyzed by ultra-performance liquid chromatography coupled with electro spray ionization mass spectrometry (UPLC-ESI-MS). Food supplements that are analyzed are largely used by beekeepers without knowing if they are benefic or not, toxic or not on the health of bees.

Furthermore the results of the analysis shows that what is represented on the labels of products could be not exactly the same amounts of the real product inside the package. It shows also that some products may be considered as benefic or as toxic depending on the amount of these micronutrients.

To continue this work in order to confirm these hypothesis, in vitro tests could be done on honeybees using these products analyzed in this thesis.

Through the obtained results we could observe that the high amount of free amino acids presented in the studied supplements does not necessarily reflect a good source of nutrients, considering that for the bee it is important to have access to a diverse set of amino acids. Bee's nutritional requirements require 10 essential amino acids (Arg, Phe, His, Ile, Leu, Lys, Met, Thr, Trp and Val). The supplement P12 appears as the richest and most balanced, followed by P05. The C08 supplement, although containing an adequate proportion in most amino acids, has an excessive amount of Arg, which may cause adverse effects.

Regarding fatty acids, the samples presented several compounds, among which the most abundant were hexanoic acids, 9-octadecenoic acid (oleic acid) and 9,12-octadecadenoic acid (linoleic acid).

Although the role of fatty acids in bee nutrition is not yet fully understood, compounds such as linoleic acid, linolenic acid, myristic acid and dodecanoic acid appear to play an important role in inhibiting some microorganisms that affect bees, as *Paenibacillus larvae larvae* (American foulbrood). For this reason the sample P05

appears as the one with the highest nutritional quality, since it presented a higher number of fatty acids. In the analysis of minerals, it was observed that protein foods are significantly richer in micronutrients. In this work, the most common elements were potassium, sodium, calcium and magnesium, while copper and manganese appeared in some foods in small quantities. Cadmium, often an associated element with heavy metal contamination appeared in only one of the products, P05, but in very small quantities, lead was not detected in any of the supplements.

In general, there were discrepancies between the results obtained and the description available on product labels, making clear the need for further quality control of these commercial products.

# RESUMO

As abelhas melíferas (Apis mellifera L.) são os principais agentes polinizadores de inúmeras plantas e árvores frutíferas e, portanto, desempenham um papel fundamental na agricultura e, de maneira mais geral, na manutenção da biodiversidade ecológica. Como a maioria dos organismos, as abelhas precisam de uma dieta diversificada, composta de minerais, hidratos de carbono, lípidos e aminoácidos (proteínas) para sobreviver e se reproduzir. A necessidade de hidratos de carbono de uma abelha adulta é satisfeita pelo néctar produzido pelas flores e também, ocasionalmente, por meladas segregadas por insetos, enquanto o pólen de flores é a principal fonte de aminoácidos, principais constituintes das proteínas, tendo um papel preponderante no desenvolvimento de larvas e abelhas jovens, fornecendo elementos estruturais de músculos, glândulas e outros tecidos.

O declínio das populações de abelhas está ligado à escassez nutricional causada pela agricultura intensiva, que reduz a diversidade e a abundância de espécies de plantas.Juntamente com a deficiência causada por alterações climáticas adversas e com a necessidade de reduzir a mortalidade de colónias, particularmente para garantir um bom estado nutricional/saúde das abelhas, a suplementação artificial de colónias de abelhas tornou-se uma questão importante na apicultura, sendo uma prática comum e crescente entre os apicultores portugueses. Esta prática, associada à escassa regulamentação existente para este tipo de produtos, está a aumentar a oferta comercial destes produtos.O impacto destes produtos nas colmeias pode permitir ao apicultor remediar desequilíbrios existentes nas colónias, resultantes de condições adversas ou de apicultura, mas também pode representar riscos à saúde da abelha e à qualidade dos produtos apícolas, dependendo das matérias-primas usadas e da presença de substâncias nocivas.

O presente trabalho, inserido no projeto ApisCibus - Alimentos artificiais para abelhas: levantamento de qualidade, digestibilidade e desempenho sobre a colmeia, teve como objetivo principal avaliar a qualidade de suplementos artificiais de

abelhas comerciais através da análise da sua composição química. Os parâmetros de qualidade avaliados foram: minerais, quantificados por espectroscopia de absorção atómica, ácidos gordos,

Analisados por cromatografia gasosa acoplada a espectrometria de massas (GC-MS) e aminoácidos analisados por cromatografia líquida de ultra-pressão acoplada à espectrometria de massas por ionização por eletrospray (UPLC- ESI-MS).Através dos resultados obtidos pudemos observar que a elevada quantidade de aminoácidos livres apresentada nos suplementos estudados, não reflete necessariamente uma boa fonte de nutrientes, considerando que para a abelha é importante ter acesso a um conjunto de aminoácidos diversificados. As exigências nutricionais da abelha requerem 10 aminoácidos essenciais (Arg, Phe, His, Ile, Leu, Lys, Met, Thr, Trp e Val).O suplemento P12 surge como o mais rico e equilibrado, seguindo-se o P05. O suplemento C08, apesar de conter uma proporção adequada na maioria dos aminoácidos, apresenta uma quantidade excessiva de Arg, o que poderá provocar efeitos adversos. Relativamente aos ácidos gordos, as amostras apresentaram diversos compostos, entre os mais abundantes os ácidos hexanóico, ácido 9-octadecenóico (ácido oleico) e ácido 9,12-octadecadienóico (ácido linoleico). Embora o papel dos ácidos gordos na nutrição das abelhas ainda não seja totalmente compreendido, compostos

como o ácido linoleico, ácido linolénico, ácido mirístico e ácido dodecanóico parecem ter um papel importante na inibição de alguns microorganismos que afectam as abelhas, como Paenibacillus larvae larvae (Loque Americana). Por esta razão a amostra P05 surge como a de maior qualidade nutricional, já que apresentou um maior número de ácidos gordos. Na análise dos minerais, observou-se que os alimentos proteicos são significativamente mais ricos em micronutrientes. Neste trabalho, os elementos mais comuns foram o potássio, sódio, cálcio e magnésio, enquanto cobre e o manganês surgiram em alguns alimentos em pequenas quantidades. O cádmio, um elemento associado frequentemente com a contaminação por metais pesados surgiu apenas num

dos alimentos, P05, mas em quantidades muito reduzidas, já o chumbo não foi detetado em nenhuma dos suplementos.

No geral, verificaram-se discrepâncias entre os resultados obtidos e a descrição disponível nos rótulos dos produtos, tornando-se evidente a necessidade de um maior controlo de qualidade destes produtos comerciais.

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# PREFACE

The Sector of Apiculture is already considered a key agricultural activity in Portugal, not only a reason for the direct impact and net contribution of honey production, but also for the services traded in maintaining biodiversity and pollinating crops. Currently, the activity is carried out by more than 10 000 beekeepers who manage more than 620 000 colonies. The development of this sector and its professionalization have resulted in a search for new beekeeping practices, not exclusively based on bee swarm maintenance, but introducing new procedures, which by changing the biodynamics of the colony, the profitability of the activity its maximized and the functionalities of the hive are multiplied. As a result, it is essential to monitor the nutritional status of a colony, which is recognized as a key factor in ensuring the bee's health. An insufficient flow of nectar and pollen into the hive immediately conditions its development, allowing the proliferation of pathogens and consequently the reduction of the activity and quantity of bees in the colonies. It is therefore natural for beekeepers to look for dietary supplements as adjuvants in hive management and to overcome nutritional imbalances. This work was done in the frame of the project ApisCibus - Artificial food for honeybees: quality survey, digestibility and performance on the bee hive (PAN 2017-2019), which the main objective was to evaluate the food supplements for bees available in the market in order to support beekeepers decision on the basis of an effective knowledge of the qualitative composition of the products and the needs of the colony. This work pretend to evaluate the quality of commercial honeybee artificial supplementation through composition analysis of commercial supplements.The honeybee artificial supplements quality will be evaluated through some nutritional parameters: determination of the mineral content (calcium, magnesium, potassium, sodium, iron, zinc, copper, manganese, lead and cadmium) using atomic absorption spectroscopy; fatty acids content through gas chromatography coupled with mass spectrometry (GC-MS); free amino acids content analyzed and quantified by ultra-performance liquid chromatography coupled with electro spray ionization mass spectrometry (UPLC-ESI-MS).

# CHAPTER 1: LITERATURE REVIEW

# 1 LITERATURE REVIEW

## 1.1 Apis mellifera

Honey bees (genus *Apis*) are social insects in the family Apidae, order Hymenoptera; they are among the Aculeata (i.e., those having stingers). They evolved after the separation of the contains 11 known species. The most important species to humans is *Apis mellifera*, Figure 1, which has been introduced all over the world for use in beekeeping, is the source of most of the world's honey [1]. Americas and Australia from Eurasia/Africa and are native only in the Old World.

**Figure 1. Worker honey bee (*Apis mellifera*) on brood comb**

The genus *Apis* is native throughout Africa, the middle East, Europe and except the far north regions, resulting in the generation of five distinct evolutionary lineages through successive colonization: African (A), Western European (M), Eastern European (C), Middle East (O) and Ethiopian (Y). Among these numerous lineages, the result of the integration between the lineages M and A resulted in the *Apis mellifera iberiensis*, which together populate the entire Iberian Peninsula [2].

## 1.2 Honeybee's organization

The honey bees spend their entire lives in social colonies. Normally, each swarm is composed between 20 000 and 50 000 species [3]. It is a very well organized society with immature individuals and adults. Immature individuals include larvae and nymphs. They are gathered in the cells of a particular area of the hive called the brood, which is the nest of the colony. Adult honeybees are divided in three different types of individuals or castes in the colony: queens, drones and workers. Each caste has its special function in the colony. The workers are undeveloped females, the drones the males and the queen is the fully developed female. The queen's job is to lay eggs, as many as several hundred in a day. These larvae develop into drones, workers or new queens, depending on how the workers treat them [4].

### 1.2.1 The queen bee

The queen, a true mother bee, is the only female that is completely developed sexually. This is a result of a full diet on royal jelly during a developmental period. She is easily distinguished from the other members of the colony by her longer body, slender appearance and lack of structures to collect pollen or functional wax glands. The main activity of the queen is to lay eggs and to keep the workers uninterested in reproduction through pheromonal control. If the queen stops producing pheromone or laying eggs, one of her most recent eggs will be moved to a specially prepared queen cell to produce a new queen. The queen is constantly attended and fed royal jelly by the workers. In the colony, she can be observed in the area of the brood nest. A queen leaves the hive three to six days after birth for mating. She takes a number of such flights over a period of 2–3 days and may mate with ten or more different drones. The sperm is stored in a special organ, the spermatheca, and the queen never mates again after this period. About 5 days after taking her mating flights, the queen begins to lay eggs. During favorable periods, a

good queen can lay more than 1500 eggs per day, which she does throughout most of her life, usually 2 to 5 years [5]. If an egg is fertilized, it will develop into a female bee (queen or worker), but if not fertilized, a male bee will result. Consequently, drones have only one set of chromosomes (haploid) acquired from the queen. When the sperm supply begins to be depleted, the workers prepare to replace her. Factors which affect egg laying are the weather, the nectar and pollen flows, the size of the queen and the condition of the colony. The number of eggs laid varies with the annual cycle as available resources of nectar and pollen vary. Large amounts of incoming resources stimulate workers to give the queen more food, which in turn stimulates her to lay more eggs [6].

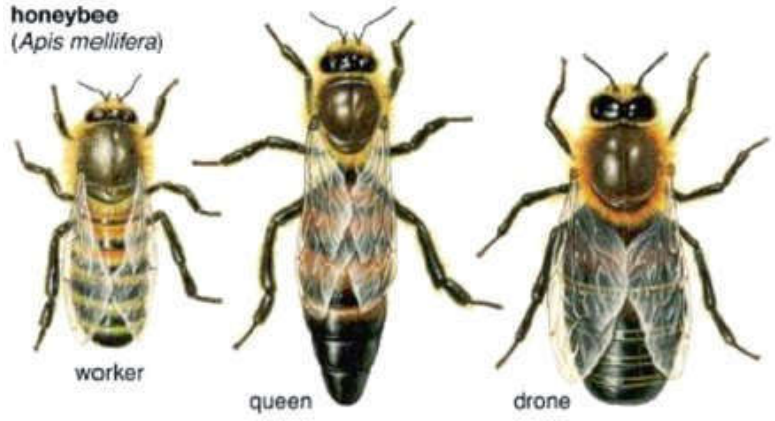


**Figure 2 Honey bee castes**

### 1.2.2 Workers

The workers are the smallest and the most numerous individuals in the colony. They are not fully developed sexually and under normal hive conditions they do not lay eggs, unlike the queen bee. This difference results from its feeding in the larval development phase, which from the third day, is switched from royal jelly to honey and pollen [6]. Worker bees have specialized structures, such as

brood food glands, scent glands, wax glands, and pollen baskets, which allow them to perform all the tasks of the hive according to the age, glandular development and needs of the hive. Thus, during their life, each bee will participated in all the tasks necessary in the hive, and if other needs arise the tasks will change accordingly because workers can change their function to meet the demands of the colony. A worker stays approximately twenty one days in the hive, with the following tasks:

- From the first to the fifth day: clean the cells, warms the brood nest and feed older larvae with honey and pollen;

- From the sixth to the ninth day: attending the queen and feed younger larvae with royal jelly;

- From the tenth to eighteenth day: store pollen, ripen nectar, clean the hive, produce wax and comb building

- From nineteen and twenty day: guard and ventilate the hive, take exercise and orientation flights to learn to fly and locate the hive;- For twenty days at most, the bee will have an activity out of the hive: orientation flights, search for nectar, honeydew, pollen, water, propolis, attack in case of aggression of the hive, looting.

These durations are variable according to the needs. In case of strong honeydew, for example, the time spent in the hive is considerably shortened [7].

After 3 weeks, the workers become foragers, gathering pollen, nectar, water, or propolis for the colony, which is used to close up openings as cement, defend the hive from all improper intrusion.

The schedule of worker bee activities is highly flexible and depends on physiological, ecological, and behavioral factors. During autumn, a reduction in brood rearing and an increase in pollen consumption result in a population of long-lived “winter” bees having increased fat bodies and protein reserves. The normal 6-week adult life of “summer” bees may be extended to several months in these “winter” bees.

### 1.2.3 Drones

Drones, the males of the colony are produced from unfertilized eggs. The

drones are the largest bees in the colony. The drone's head is much larger than that of either the queen's or the worker's. The eyes are large and cover practically the whole head. The end of the abdomen is blunt and is covered with a tuft of small hairs. Drones have no sting, pollen baskets, or wax glands since they are designed for mating only. Drones take their first flights at about 8 days old and are sexually mature at 12 days old. Only a few of them are tolerated in the hive at spring and fall, more in the summer, but none in the winter. In fact, the workers keep them out of the hive to starve to death in the autumn. The life span of a drone is from two to three months [8, 9].

### 1.3 Honeybee development and differentiation

The development of the three bee castes, queen, workers and drones, undergoes in four stages: egg, larva, pupa and adults, Figure 3.

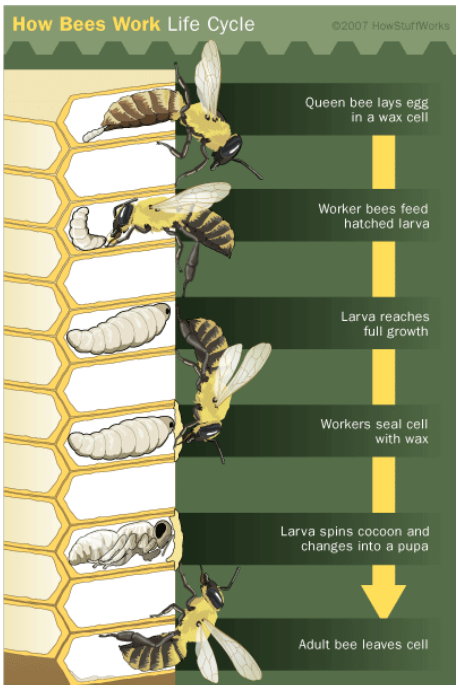


Figure 3 The biological cycle of honeybees

The first stage in the development of a bee occurs when the queen deposits a

single egg at the bottom of a cell. She can lay fertilized and unfertilized eggs: fertilized eggs can give workers or queens, however, the other eggs usually give drones. The newly egg measure 1 to 1.5 mm long and is shaped like an ellipsoid. It is elongated, rounded at the ends, and slightly convex in the center [10].

After three days, the egg hatches into a first-instar larva and is then fed regularly by nurse bees. Honey bee eggs can be difficult to see, but their presence indicates a laying queen is present in the colony [10]. Larvae are white and lay in a curled "C" shape at the bottom of their wax cell. After successive stages of growth and molts, the larva completely covers the floor of its cell, and then changes position, stretching out along the depth of the cell. During its life, the larva goes through five larval instars. The number of days a honey bee spends as a larvae varies by caste (worker: 6 days, drone: 6.5 days, queen: 5.5 days).A worker larva fed exclusively on royal jelly can give a queen. Worker larvae from the 4th day are fed with nectar and small amounts of pollen. This diet is low in fat ,in contrast with a protein diet rich in nitrogen compounds, which does not interfere with larval development, but it can causes a clear delay in the formation of reproductive organs, being these food deficit a clear nutrient castration. Male's larvae receive from the fourth day honey and pollen which seems richer in nitrogen [11].

When the mature larvae are ready to molt into pupae they extend their bodies into an upright position in the cell, and adult workers tending to cover the brood, the pre-pupal larvae with a wax capping. Beneath the wax capping, pre-pupal honey bee larvae molt into pupae, Figure 3. The pupa has all the adult bee's distinct body parts, but they all adhere tightly to the body, and some appendages are not yet fully expanded. Before emerging, the pupa becomes gradually darker in color. Finally, transformed into an adult, it slowly chews its way out of the cell. In *A. mellifera*, the complete metamorphosis from newly laid egg to emerging adult worker requires 21 days: three as an egg, six as a larva, and 12 as pupae [10].

## 1.4 Honeybee nutrition

Most organisms need a diverse diet consisting of minerals, carbohydrates (sugars), fats, and amino acids (proteins) to survive and reproduce. The three honey bee castes have somewhat different nutritional needs and feeding mechanisms to satisfy their food requirements, as do the larval and adult stages within each caste. But the starting materials for brood and adult bees, whether workers, queens, or drones, are the same: nectar and pollen. These two floral products provide all of the food necessary for larval growth and metamorphosis and for adult development and functions [1].

Nectar (produced by the glands of flowers to attract pollinators) is rich in sugar and becomes the bee's main energy source. Flower pollen is the main source of amino acids that make up proteins (and some fats) that provide the building blocks in the bee's body. Honey bees are willing to eat fresh nectar (which has far more water and less sugar than honey) and pollen as it comes into the hive. However, altering nectar and pollen gives them ability in storing these products for those inevitable times when pollen and nectar become scarce in early spring and late fall. Basically, nectar provides carbohydrates in the form of sugars, and pollen provides protein, lipids, vitamins, and minerals [12].

To preserve it from deleterious microorganisms, honeybees mix pollen with nectar, honey or other glandular secretions. This is could be by inoculating the pollen with microorganisms that reduce the pH through fermentation [13].

### 1.4.1 Carbohydrates

Carbohydrates are the most widely distributed and abundant organic compounds on earth. They have a huge role in metabolisms of both of animals and plants. Carbohydrates biosynthesis is obtained by green plants by carbon dioxide and water in the presence of light energy [14].

These macronutrients are used by honeybees as an energy source. The

carbohydrate requirement of adult honey bees is satisfied by plant-produced sugars, primarily from nectar produced in flowers but also occasionally from extra floral nectarines or honeydew secreted by plant-feeding insects [1]. Sugar content in nectar ranges from 4 % to 60 % or higher depending upon the floral source and a number of environmental conditions such as temperature and humidity [1]. Sucrose, glucose, and fructose are the main sugars found in nectar, and nectars can be broadly classified into three groups: predominately or totally sucrose, approximately equal proportions of sucrose, glucose, and fructose, and predominately glucose or fructose or the both of these two compounds. The proportions of these three sugars depend on the plant species from which nectar is collected [12].

In general, honey bees prefer nectar with a sugar concentration of 30 to 50%, which seems to elicit the maximum collection response by honeybees, but in the field, they collect nectar of varying water content, depending on what is available in their local environment [12]. The optimal concentration for energy intake is about 60% for honey bees; above this, nectars become too viscous for efficient licking [12]. In addition to these three sugars, α-methyl glucoside, maltose, trehalose, and melezitose have also a nutritional value to bees. Most other sugars neither taste sweet nor have nutritional value [1].

Often, the nectar collected by foraging workers can be fed to brood and adults directly, but it is usually processed into honey first. The nectar is carried back to the nest in the honey stomach and transferred to nest workers for processing. The nectar is manipulated and enzymatically converted to honey by bees. These enzymes break down the sugars into simple inverted forms, which are the most easily digestible by bees and also protect the stored honey from bacterial attack. The nectar is then processed and placed into cells for further evaporation by fanning, where the water content is reduced to less than 18% to protect the nectar from yeasts. When the enzymatic activity and water evaporation are complete, the nectar is considered to be "ripened" and can be called honey, which is sealed beneath a wax capping until

required for feeding to larvae or adults [1].

A worker bee needs 11 mg of dry sugar each day. This translates to about 22 μL of 50% sugar syrup per worker per day [15]. A worker larva requires about 142 mg of honey for development, and the annual honey requirements for a colony have been estimated at about 60-80 kg [1].

Although nectar and honey satisfy the carbohydrates requirement of adult honey bees, it is interesting that pollen and bee bread contain 30-35% of sugar. In bee collected pollen, the level of non-reducing sugar averages 2.71% and reducing sugars average between 18 and 41% [21].

Researchers tested 34 carbohydrates and related compounds and found that honeybees consider only seven of them sweet. Sugars which do not taste sweet to honey bees are of little or no nutritional value to them [16]. When given a choice, honeybees show a preference for, sucrose, glucose, fructose and maltose, when tested in laboratory assays mixtures of sugars did not have an additive effect. It was found that glucose and fructose were only half as attractive as sucrose to foraging bees when they were allowed to choose among the three sugars [16]. Caged honeybees preferred diets containing sucrose to those containing sugar combinations, honey, invert sugar or isomerized corn syrup. It was found that sucrose was superior to other sugars in both acceptance and nutritive value when they fed 13 sugars to honeybees and measured survival, water and sugar consumption [16].

Although honeybees can use many sugars in their diets, there are also several sugars that are either toxic or useless to honeybees because they lack the proper enzymes for digestion. Mannose, lactose, galactose and raffinose either are toxic to honeybees or reduce their longevity. Mannose is especially toxic and can kill honeybees within in few minutes of feeding [15].

### 1.4.2 Proteins

Proteins are important constituents of food: For the honeybees, flower pollen is the main source of amino acids that make up proteins (and some lipids) that provide the building blocks in the bee's body. It is largely used to feed developing larvae and young bees to provide structural elements of muscles, glands and other tissues. It is also used in the production of royal jelly, the specially food produced by worker bees to feed the queen, developing queen larvae, and worker larvae up to 72 hours of age [12].

Pollen is the male germ of plants. Forager honey bees collect pollen from anthers of flowers and pack it on the corbiculae, specialized structures on the hind legs. In the process of collecting pollen, bees inadvertently carry out the function of pollination of the various plants they visit [16]. Pollens may contain from 6 to 28% protein [17]. Pollen is made up of other various substances, including fats, lipids, carbohydrates, vitamins, minerals and others [18].

Honey bees need to ingest ten amino acids described as essential to their diet [19] which are: arginine, histidine, lysine, tryptophane, phenylalalanine, methionine, threonine, leucine, isoleucine, and valine [18]. Amino acid requirements are highest for L-leucine, L-isoleucine and L-valine, and limitations of one of the essential amino acids in the food protein limit colony development. Pollens from different plants have different nutritive values for bees [16]. The amino acid composition of different pollens have been investigated by numerous researchers, which investigated the pollen from five different plant sources and also mixed pollen samples, and the found that the 10 essential amino acids are always present in almost the same quantity. It was observed that in general honeybees visit more frequently plants with a larger number of amino acids than those that have a lower number [21].

Colonies collect 10–26 kg of pollen per year [22]. In contrast to honey, only a small amount of pollen is stored in the colony at any given time, and stores quickly diminish during non-foraging periods. It has been demonstrated that the longevity of

worker bees is greatly enhanced when they receive a diet of high protein pollen, as compared to diets dominated with pollens with low protein levels [16].

### 1.4.3 Lipids

Lipids are formed with structural units with a pronounced hydro solubility. The majority of lipids are derivatives of fatty acids. Fatty acids are weak organic acids, which have only one carboxylic function. They seldom exist in the free state in nature, and they are essentially in the esterified form [14].

Generally, fatty acids have an aliphatic carbon chain, hydrophobic, and even-numbered (4 to 24 atoms). Depending on the number of carbons, we have short (4 to 8), medium (10 to 14) or long fatty acids (16 to 24). A fatty acid chain can contain up to six double bonds or instaurations. This distinguishes three classes of fatty acids: saturated fatty acids (SFA), monounsaturated (MFA) and polyunsaturated (PFA) [20].

Lipids, such as fatty acids and sterols, are important to honey bees primarily, as a source of energy, with an important role in development and reproduction. The main source of lipids is pollen and which contains 1 to 20% (usually less than 5%). Most pollen contains less than 0.5% sterols, but these are required for bee metabolism since bees cannot synthesize cholesterol without the precursors obtained from pollen. Nearly all insects need to obtain sterol from their diet because of their inability to synthesize them directly. Sterols are the precursor for important hormones such as larvae hormone. Workers honeybees convert ingested phytosterols to 24- methylene cholesterol (the honey bees' major sterol), sitosterol and isofucosterol [16]. Besides sterols, is the dietary needs of honeybee lipids are unclear. Most lipids present in the hemolymph are associated with the synthesis of single lipoprotein called lipophorin, in the hemolymph, which as a role of carrier between the intestine and the fat in the body and wing muscles. The majority of lipids are stored in the form of triacylglycerol [21].

There are two other functions that appear to be possible in explaining the role of lipids in pollen. Certain lipids in pollen would appear to act as strong attractants to foraging honey bees and some fatty acids exhibit significant antimicrobial

activity [16]. It has been observed that foraging honey bees prefer pollens with high lipid levels over those pollens with lower lipid levels. The addition of either whole pollen lipids or the soluble fraction extracted in cold acetone significantly increased the amount of dietary supplement consumed by caged honey bees [21]. The addition of the insoluble fraction, or an extract of the volatile substance in pollen, led to decreased food consumption. This indicated that the addition of fat to artificial diets may be beneficial or detrimental, depending on the composition and quantity of the individual components of the lipids. The total lipid concentration within a pollen supplement is recommended to be 5%–8% [15, 16].

### 1.4.4 Vitamins

Although they are essential for all animals, not a great deal is known about the vitamin requirements of honey bees.

Pollen has been demonstrated as an excellent source of these vitamins, more generally rich in water-soluble vitamins and poor in the fat soluble vitamins. In general, the vitamin requirements of honeybees are satisfied as long as the pollen or supplementary protein foods are nutritionally adequate abundant, and available in the hive.

Normally the pollen contains the seven B-complex vitamins (thiamine, riboflavin, pyridoxun, pantothenic acid, niacin, folic acid and biotin) which are essential for most insects. Inisitol, ascorbic acid and also other water soluble vitamins are present in pollen which has been demonstrated as an excellent source of these micronutrients [15]. Nurse bees are thought to need the following vitamin B complex for brood rearing: thiamine, riboflavin, nicotinamide, pyridoxine, pantothenic acid, folic acid, and biotin. A mixture of the fat soluble vitamins A, D, E and K in the diet substantially improved the amount of brood produced, although these vitamins are not regarded essential [16]. Like sterol and lipids, the vitamin needs of a honey bee colony are satisfied if pollen stores are abundant in the hive or fresh pollen is being

brought into the colony. It is not known whether micro-organisms naturally present in the alimentary canal of bees may play a role in providing vitamins and other essential substances [15].

### 1.4.5 Minerals

Minerals are the constituents which remain as ash after the incineration of plant and animals tissues. There are various minerals in nature ( Ca , P , K , Cl , Na , Mg , I , Mo ,etc.). According to their biological roles, they can be divided into essential elements, for which the biological roles are known, non-essential elements with unknown functions, and toxic elements, which may be ingested through food intake or from water absorbed from the air [14].

Little is known about the mineral requirements of honey bees.. All the required minerals can be obtained from pollen, although nectar also contains minerals, including potassium, magnesium, calcium, sodium, iron, copper, manganese, zinc, aluminum, cadmium, chromium, lead, nickel and selenium, although many elements are only present as trace amounts. Pollen contains normally between 2 to 4% ash on a dry weight basis, or between 1 to 7% of minerals, being the most common potassium, phosphorus, calcium, magnesium and iron [18]. Excessive levels of sodium, sodium chloride, and calcium have been shown to be toxic to honey bees. The optimal ash concentration for maximum brood rearing seems to be at 0.5%–1%. Pollen with more than 2% ash inhibits brood production [15]. Although these inorganic elements are present in pollen in relatively small amounts, they are essential to such life process as enzyme systems. Mineral requirements of honeybees have not been established by nutritional studies because of the difficulty of preparing experimental diets deficient in trace minerals. Most often diets containing minerals have been based on the levels occurring in pollen, however, since pollen composition is highly variable, quantitative analyses of it may provide valuable information about the mineral requirement of honeybees [6].

### 1.4.6 Water

Honey bees forage water for two purposes. One is to use it to dilute honey so that it can be used as brood food. The second is to use water to regulate the temperature in the hive, cooling by fanning over a thin layer of water when the ambient temperature is over 35°C. During winter time, bees have enough water from condensation inside the hive. When bees have a choice, they usually prefer water with some salts. Other species of honey bees (e.g. *Apisdorsata*, *A. cerana*) have been observed to forage on urinals or open restrooms in Asia. This is probably because bees are not obtaining adequate sodium from their normal diet [15].

## 1.5 Artificial feeding

### 1.5.1 Energy supplementation

Bee-population declines are linked to nutritional shortages caused by land-use intensification, which reduces diversity and abundance of host-plant species. Bees require nectar and pollen floral resources that provide necessary carbohydrates, proteins, lipids, and micronutrients for survival, reproduction, and resilience to stress [15]. In periods of scarcity of nectar the colonies become more aggressive in the defense of the hive, at the same time there is a decline in the demand for pollen, since the amount of energy available to carry out the flights is smaller, also decreasing their hygienic behavior and altering the functioning of the colony. The application of energy supplementation in these situations may maintain the brood area and the population of the colony at a high level or even provoke an expansion stimulus. Some researchers recommend the use of an energy food whenever the hives have less than two frames of reserves (approximately 6-8 kg of honey) [21].

The identification of which sugar diet is best suited for bee feeding is a controversial subject. In a former study, it was observed that bees work better when

they are fed with sucrose compared to fructose [21].Rogers (1995) [23], states that high levels of fructose can produce hydrolyzed acids and enzymes, which can be lethal to bees .Some tests performed by Pesante (1992) [24] providing syrup-based feed with 50% sucrose showed very positive results in the development of the hive. For this same feed and for it to be possible the construction of combs and production of honey, it is necessary to consume 37 kg of sugar within a period of 51 days. One of the energetic foods that have been widely used is inverted sugar, however, several results indicate that during the breakdown of sucrose into glucose and fructose by the action of the added acids, hydroxyl methyl furfural (HMF) is release, a compound that is quite harmful to bees because it causes intestinal ulcer and dysentery [21].

### 1.5.2 Protein supplementation

Beekeepers often feed colonies of honeybee artificially during periods of pollen and nectar deficit and it depends on the brood-rearing activity and nutritional state of the colony, the quantity and quality of incoming pollen and nectar, and the food reserves in the hive [21]. The supply of proteins is particularly necessary when it is intended to maintain or increase the amount of new bees or when the available pollen in the field becomes limited, which usually occurs before or during a nectar flow. The amount and time at which this supplement should be applied depends on the strength of the colony, the desired level of production, the attractiveness of the supplement and also its effectiveness [13].

Protein supplements containing pollen are generally better accepted than the ones without pollen, but it should be noted that the nutritive properties of pollen when stored for a long time may degrade. Although pollen is undoubtedly the most efficient protein food, studies are looking for an alternative source of protein to meet the needs of bees, since providing pollen-rich food as a supplement is economically unfeasible [21]. According to (TABER, 1963) [25] the bees can be

fed artificially with a mixture of pollen, granulated sugar, brewer's yeast and water or still using soybean meal and milk powder, however some studies have considered soy flour and powdered milk as presenting toxicity to the bees [21]. The results obtained by BARKER (1997) [26] indicated that 40% of the sugars contained in soybeans are toxic to bees and that the addition of 10% lactose or galactose increases worker mortality and reduces the acceptability of sugar syrup provided.

# CHAPTER 2: MATERIALS AND METHODOLOGY

# 2 MATERIALS AND METHODOLOGY

## 2.1 Honeybee's food supplements samples

For this study, 43 honeybee's food supplements found in the market were analyzed, 20 of them were classified as energetic and 14 were considered as protein supplements and the 9 where considered both energetic and protean supplements in the same time. Before samples analyses, they were lyophilized and preserved in a desiccator.

The table 1 classified the products as energetic or protein supplements:

**Table 1-classification of samples as energetic or protein supplements**

| Code of the commercial food | Classification | |
|---|---|---|
| | Energitic supplements | Protein supplements |
| E01 | X | |
| E02 | X | |
| E03 | X | |
| E04 | X | |
| E06 | X | |
| E07 | X | |
| E08 | X | |
| E09 | X | |
| E10 | X | |
| E11 | X | |
| E12 | X | |
| E14 | X | |
| E15 | X | |
| E19 | X | |
| EP01 | | X |
| EP02 | | X |
| P03 | | X |

| | | |
|---|---|---|
| P04 | | X |
| P05 | | X |
| P06 | | X |
| P07 | | X |
| P09 | | X |
| P10 | | X |
| P11 | | X |
| P15 | | X |
| P16 | | X |
| P17 | | X |
| PC01 | | X |
| C02 | X | X |
| C03 | X | X |
| C04 | X | X |
| C05 | X | X |
| C06 | X | X |
| C08 | X | X |
| C09 | X | X |
| C11 | X | X |
| C12 | X | X |
| ES01 | X | |
| ES02 | X | |
| ES03 | X | |
| EC01 | X | |
| EC02 | X | |
| EC03 | X | |

## 2.2 Fatty acids analysys

### 2.2.1 Fatty acids extraction

A sample (2g) was treated with petroleum ether in a Soxhlet apparatus for 4h.

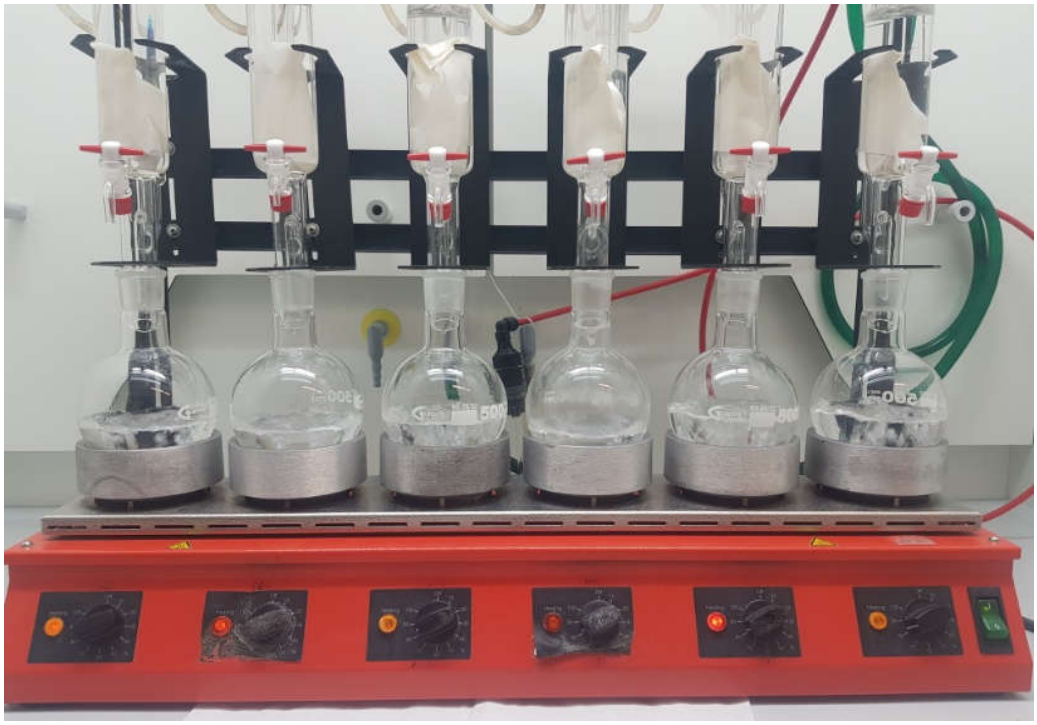

**Figure 4 Soxhlet apparatus**

The petroleum ether extract was then evaporated under reduced pressure to dryness, the residue was weighed and the total fatty acids content was expressed as a mass percentage. The analysis was performed in triplicate. The extract was kept at -20 º C for the fatty acids analysis by GC-MS.

### 2.2.2 Determination of the fatty acids composition by GC-MS

Fatty acids were determined by gas-liquid chromatography with mass spectrometry detection (GC-MS) based on the following trans-esterification procedure: fatty acids were methylated with 4450 µL of methanol: sulfuric acid: toluene 2:1:1 (v:v) and 550 µL of internal standard (pentanoic acid; 0,5 mg/mL), for at least 12 h in a bath at 50 °C and 160 rpm; then, 3 mL of deionized water was added, to obtain phase separation; the FAMEs were recovered with 3 mL of diethyl ether by shaking in vortex, and the upper phase was passed through a micro column of sodium sulfate anhydrous to

eliminate the water; the sample was recovered in a vial with Teflon, and before injection, the sample was filtered with 0.2 µm nylon filter.

Figure 5: Gas-chromatography coupled with mass spectrometry (GC-MS)

The fatty acid profile was analyzed with a Perkin Elmer system (GC Clarus ® 580 GC

module and Clarus ® SQ 8 S MS module) gas chromatograph, equipped with DB-WAX fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J &W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 6.1, Perkin Elmer, Shelton, CT, USA). Oven temperature was programmed, 50°C for 1 minute, 50-200°C, at 25°C/min, subsequently at 3°C/min up to 230°C, and then held isothermal for 23 min. The transfer line temperature was 250°C; ion source temperature, 230°C; carrier gas, helium, adjusted to a linear velocity of 1mL/min; ionization energy, 70 eV; scan range, 40- 300 u; scan time, 1 s. Split injection (1:50) was carried out at 250 °C. For each analysis, 1 µL of the sample was injected in GC. Identifications were based on the comparison of the obtained spectra with those of the NIST mass spectral library and were confirmed using linear retention indices determined from the retention times of an n-alkane (C7–C40) (Supelco, Bellefonte, PA, USA) mixture analyzed under identical conditions, with comparison with published

data, and when possible with commercial standard compounds. Quantitation (average value for two replicates per sample) was carried out using relative values directly obtained from peak total ion current (TIC).

## 2.3 Amino acids analysis

### 2.3.1 Sample preparation for amino acid extraction:

From each sample were taken 5.0g which were added in flasks containing 10 mL of water: acetonitrile (50:50) (v/v) and 3.0 mM N-Acetyl-L-Tyrosine (as an internal standard) solution. The mixture was shaken with vortex for 5 min, then sonicated for 10 min at room temperature (20 °C). The samples were immediately centrifuged at 4°C at 10.000 rpm for 10 min (Heraeus Multifuge X1R – Thermo Fisher Scientific). The supernatant was filtered through 0.2 µm nylon membrane filter (Whatman PURADISC 25 NYL) and stored at -4 °C.

Figure 6 The UPLC–MS/MS instrument

### 2.3.2.Chromatographic condition for UPLC–MS/MS amino acid analysis

The UPLC–MS/MS instrument consisted of a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, USA) equipped with a diode-array detector and coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an autosampler maintained at 5 °C, a degasser, a photodiode-array detector, an automatic thermostatic column compartment. The chromatographic separation was carried out on a U-VDSpher PUR C18-E 100mm×2.0 mm id, 1.8 µm column (VDS optilab, Germany) and its temperature was maintained at 40 °C. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile/water (50:50, v/v), which were previously degassed and filtrated. A multistep gradient program at a flow rate of 0.400 mL/min was used (Table 2) and the injection volume was 5 µL.

MS detection was performed in positive ion mode by multiple reaction monitoring (MRM) using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA,USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 5.5 kV, a source temperature of 400°C, a capillary voltage of 18 V. The tube lens offset was kept at a voltage of 25 V. Mass spectra were acquired by full range acquisition covering 100–1500 m/z. The collision energy used varied from 14 to 30 (arbitrary units)depending on amino acid investigated (Table 3). Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA).

**Table-2 Chromatographic conditions**

| Time (min) | Flow (mL/min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|---|
| 0.5 | 0,400 | 80 | 20 |
| 0.5 | 0,400 | 80 | 20 |
| 0.75 | 0,400 | 70 | 30 |
| 1.00 | 0,400 | 70 | 30 |
| 5.00 | 0,400 | 5 | 95 |
| 6.00 | 0,400 | 5 | 95 |
| 8.00 | 0,400 | 80 | 20 |
| 10.00 | 0,400 | 80 | 20 |

**Table- 3 Chromatographic and MRM method parameters for free amino acids using UPLC–MS/MS**

| Amino acid | Retention time (min) | Quantification transition (m/z) | Confirmatory transition (m/z) | Collision energy (V) |
|---|---|---|---|---|
| **Histidine (His)** | 0,62 | 156 | 137, 111, 109, 94 | 25 |
| **Lysine (Lys)** | 0,61 | 147 | 130, 129, 100 | 25 |
| **Glutamine (Gln)** | 0,61 | 147 | 129, 100, 83 | 26 |
| **Glutamic acid (Glu)** | 0,61 | 148 | 130, 129, 101, 83 | 25 |
| **Serine (Ser)** | 0,58 | 106 | 88, 87, 85, 59 | 25 |
| **Alanine (Ala)** | 0,65 | 90 | 68, 61 | 18 |
| **Glycine (Gly)** | 0,65 | 76 | 75, 47, 29 | 14 |
| **Threonine (Thr)** | 0,59 | 120 | 101, 99, 83, 73, 71, 55 | 25 |
| **Aspartic acid (Asp)** | 0,60 | 134 | 115, 87,86, 73 | 15 |
| **Valine (Val)** | 0,67 | 117 | 100, 90, 71 | 25 |
| **Methionine (Met)** | 0,76 | 150 | 132, 103, 101, 55 | 25 |
| **4-Hydroxy-Proline (Pro))** | 0,81 | 132 | 85 | 20 |
| **Isoleucine (Ile)** | 0,82 | 132 | 120, 114, 104, 86, 85, 71, 68 | 25 |
| **Leucine (Leu)** | 0,82 | 132 | 120, 114, 104, 86, 85, 71, 68 | 25 |
| **Asparagine (Asn)** | 0,82 | 133 | 115, 112, 104, 87, 89, 85 | 25 |
| **Arginine (Arg)** | 0,62 | 175 | 157, 140, 130, 115, 111, 97 | 30 |
| **Phenylalanine (Phe)** | 1,01 | 166 | 148, 130, 119 | 25 |
| **Tryptophan (Trp)** | 1,46 | 205 | 187, 159, 132 | 25 |
| **Cysteine (Cys)** | 0,60 | 121 | 98, 97, 75 | 25 |
| **Tyrosine (Tyr)** | 0,74 | 182 | 164, 135 | 25 |
| **Cistine** | 0,64 | 241 | 224, 14, 177, 168, 93, 151 | 22 |

## 2.4 Minerals analysis

For the analysis of the minerals, the following elements were evaluated: potassium (K),

sodium (Na), calcium (Ca) and magnesium (Mg) using a flame atomic absorption

spectrophotometer: Pye Unicam PU9100X. The determination of manganese (Mn), copper (Cu) and cadmium was made by atomic absorption spectrophotometry in a graphite chamber using a Perkin Elmer PinAAcle 900 spectrophotometer.

### 2.4.1 Sample digestion

A sample (1g) was weighed into a PTFE digestion tube to which was added 10mL of concentrated nitric acid ($HNO_3$). The digestion was done in a microwave using the following ramp temperature program: 15 minutes until 200ºC with a power of 1200 W. These conditions were maintained for another 15 minutes. Then, it was allowed to cool, and the sample was quantitatively transferred to a 50 mL volumetric flask.

### 2.4.2 Sample analysis

The analysis of the different minerals requires the prior preparation of specific solutions and standards according to the following procedure:

#### 2.4.2.1 Potassium and sodium

For the analysis of the potassium and sodium elements, a cesium chloride buffer (10 g/L) and the different standard solutions were prepared according to the following specification: Solution 1: 10mL of the potassium standard (1000 ppm) and 5mL of sodium standard (1000 ppm) were pipetted into a 20mL flask and the volume completed with deionized water. This stock solution was further diluted, according with Table 4, to provide the following calibration standards.

**Table-4 The calibration standards used in the spectrophotometer for the determination of the amounts of potassium and sodium**

| Standard | V(sample)/mL | $V_f$/mL |
|---|---|---|
| P1/4 | 0.25 | 50 |
| P1/2 | 0.25 | |
| P1 | 1.00 | |
| P2 | 2.00 | |
| P3 | 3.00 | |
| P4 | 4.00 | |
| P5 | 5.00 | |

The calibration standards used in the spectrophotometer resulted from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5mL CsCl buffer in a final volume of 50 mL).

For the detection of potassium in the supplement, to a 5mL of the digested supplement solution, 1mL of the buffer solution and 4mL of deionized water were added. For the detection of sodium in the supplement, to a 10mL of the digested supplement solution, 1mL of the buffer solution were added. Readings were taken according to the recommended conditions for the equipment.

#### 2.4.2.2 Calcium and magnesium

For the analysis of calcium and magnesium, a lanthanum solution (10g/L) was prepared by diluting 13.15g of $La(NO_3)_2$ in 1L of deionized water. In addition, a standard solution of Ca (1000ppm, solution 2) and a standard solution of Mg (1000ppm, solution 3) were prepared in 10mL of deionized water. Further, from stock solutions 2 and 3 a series of standard solutions were prepared according to the following Table 5.

**Table-5 The calibration standards used in the spectrophotometer for the determination of the amount of potassium and sodium**

| Standard | V(sol 2)/mL | V(sol 3)/mL | $V_f$/mL |
|---|---|---|---|
| P1/4 | 0.25 | 0.25 | 50 |
| P1/2 | 0.25 | 0.25 | |
| P1 | 1.00 | 1.00 | |
| P2 | 2.00 | 2.00 | |
| P3 | 3.00 | 3.00 | |
| P4 | 4.00 | 4.00 | |
| P5 | 5.00 | 5.00 | |

The standards used for the calibration of the spectrophotometer for the determination of Ca resulted from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5 mL of solution La to a final volume of 50 mL). The standards that are used for calibration of the spectrophotometer for determination of Mg result from the thirty-three fold dilution of these standards (1.50mL solution of each standard and 5mL of solution La to a final volume of 50mL).

For the detection of potassium in the supplement, to a 5mL of the digested supplement solution, 1mL of the buffer solution and 4mL of deionized water were added.

For the analysis, to 10 mL of each of the digested solutions, 1mL of the lanthanum solution was added. The Ca and Mg determinations were performed according to the recommended conditions for the equipment.

#### 2.4.2.3 Manganese, copper and cadmium

For the determination of manganese, a matrix modifier was used by diluting 1.7mL of a magnesium nitrate solution, $Mg(NO_3)_2$, 10g / L, and completed to a final volume of 10mL with deionized water. Two standards were then prepared for Manganese, measuring 0.50mL of standard solution (1000ppm) to a final volume of 50mL of deionized water and another by diluting 0.20mL of the previous solution to a final volume of 50mL of deionized water (standard 2). For copper, the matrix modifier was obtained by diluting 1.0mL of palladium solution, Pd, 10g/L and 0.1mL of magnesium nitrate solution, $Mg\ (NO_3)_2$ , to a final volume of 10mL of solution in deionized water. Then, two copper standards were then prepared by diluting 0.50mL of the 1000 ppm standard solution (Vf = 50 mL deionized water, standard 1) and diluting 0.50mL of the previous solution to a final volume of 50mL (standard 2).

For determination of cadmium, the matrix modifier was prepared by diluting 0.10 mL of magnesium nitrate solution, $Mg\ (NO_3)2$ , and 1.0 mL of 10% monobasic ammonium phosphate solution, $NH_4H_2PO_4$ , in 10 mL of deionized water. Two standard solutions were then prepared, the first by diluting 0.25 mL of standard solution (1000ppm) to 50mL with deionized water (standard 1) and a second, diluting 0.10

mL of the above solution to 50 mL with deionized water (standard 2). The standards used to construct the calibration curve resulted from dilution of standard 2, according to the table 6.

Table-6 The calibration standards used in the spectrophotometer for the determination of the amount of Manganese, copper and cadmium

| **Standard** | **V(P2)/mL** | **V(matrix)/mL** | **V($H_2O$)/µL** |
|---|---|---|---|
| P1/4 | 5 | 5 | 15 |
| P1/2 | 10 | 5 | 10 |
| P1 | 15 | 5 | 5 |
| P2 | 20 | 5 | 0 |

For the analysis of the samples, 20µL of sample and 5µL of matrix modifier were pipetted and the recommended instrumental conditions for the analysis of each one were used.

# CHAPTER3: RESULTS AND DICUSSION

# 3 RESULTS AND DISCUSSION

## 3.1 Results of fatty acids' analysis

The identification and quantification of fatty acids present in supplements was performed by gas chromatography, coupled to a mass detector (GC-MS). The analysis was performed on supplements that had a higher fat percentage in the nutritional analysis (data not show). Figure 7 shows a typical chromatogram obtained for the samples where 22 distinct fatty acids can be identified.

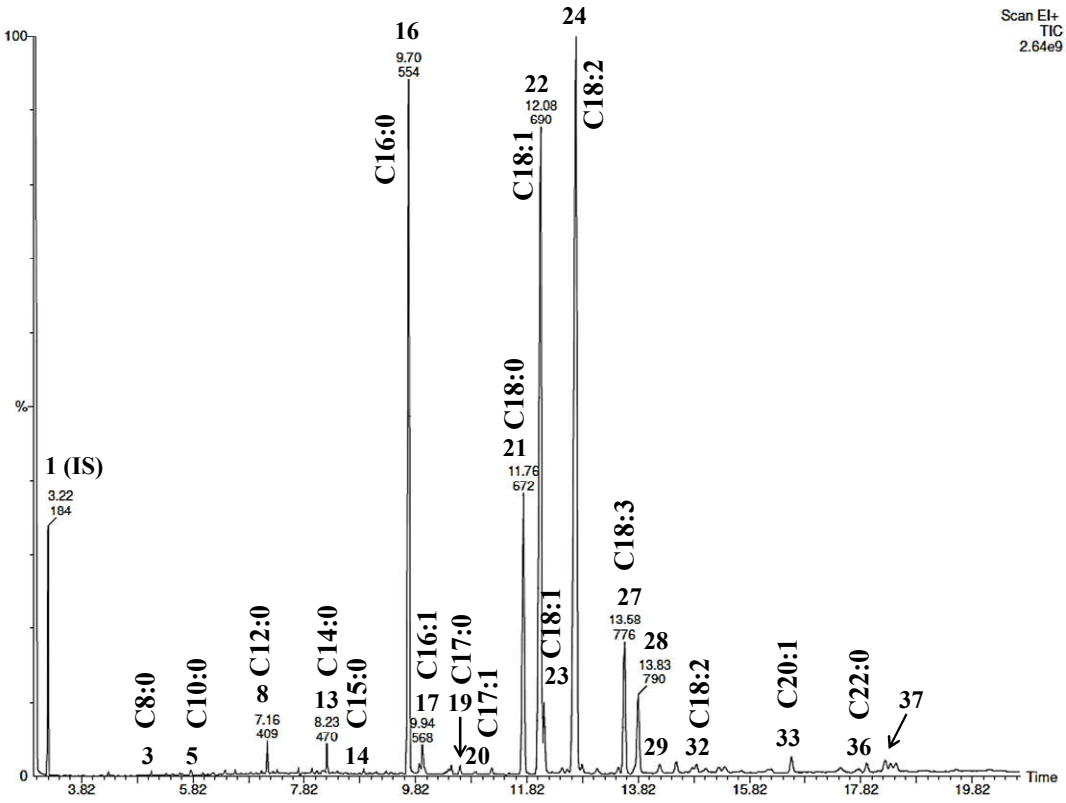


**Figure 7 Fatty acid profile for supplement P05**

The overall results for the samples can be seen in Table 7, with the highest fatty acid values found in protein foods. P05 sample among all food supplements with a higher total fatty acid, with hexadecanoic, 9-octadecenoic acid (oleic acid) and 9,12-octadecedienoic acid (linoleic acid) appearing in greater quantities. In addition to these mentioned acids, stearic acid (octadecanoic acid) and other 14- and 16-carbon saturated acids are also frequently identified in the various samples. At the same time a set of additives frequently used to prevent oxidation were identified, such as BHT and other phenol derivatives.

The role of lipids in bee's nutrition is not completed understood, but the principal source is pollen. Bee's obtained lipids exclusively through pollen and its content is generally less than 10% of dry pollen mass. However, lipid contents greater than 10% are considered attractive to bees [28] and maybe is one of the reasons to introduce lipids in the supplementts. Furthermore, scientific researches prove that pollens with high lipid concentrations and dominated by linoleic, linolenic, myristic and dodecanoic acids probably play a significant role in inhibiting the growth of the spore-forming bacteria, *Paenibacillus larvae larvae* (American foulbrood), *Melissococcus pluton* (European foulbrood) and other microbes that inhabit the brood combs of beehives [27]. Supplements with high concentration in oleic and palmitic acids probably have a greater role in honey bee nutrition. In the first hand, these two fatty acids are present in all the analyzed supplements which play a positive role in their nutritional value. In the other hand, we find these benefic fatty acids in the analysed samples and which play a significant role in the inhibition of those microorganisms. Dodecanoic acid is present in samples EC02 and P05, while linoleic acid is present in EC01, EP01, ES02, P05 and linolenic acid is present in EC01, EP01, ES02, P05. Besides that, P05 also contains oleic acid and palmitic acid. For that reason, P05 has the best nutritional quality if we compare it with EC01, EP01 and ES02.

**Table-7 Fatty acid quantification (%) performed by gas chromatography coupled to mass detector (GC-MS)**

| | | | | Samples | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Nr** | **C:D** | **Compounds** | **Commonname** | **EC01** | **EC02** | **EC03** | **EP01** | **EP02** | **ES01** | **ES02** | **ES03** | **P05** |
| **2** | C6:0 | Hexanoic acid, 2- ethyl, methyl ester | | - | - | - | 0,003±0,000 | - | - | - | - | - |
| **3** | C8:0 | Octanoicacid, methylester | Caprylicacid, methylester | - | - | - | - | - | - | - | - | 0,004±0,001 |
| **4** | C6:2 | 2,4- Hexadienoicacid, methylester | | - | - | - | - | - | - | - | - | - |
| **5** | C10:0 | Decanoicacid, methylester | Capricacid, methylester | - | - | - | - | - | - | - | - | 0,004±0,005 |
| **6** | | Benzoicacid, methylester | | - | - | - | - | - | - | - | - | - |
| **7** | C10:1 | 4-Decenoic acid, methylester | | - | - | - | - | - | - | - | - | - |
| **8** | C12:0 | Dodecanoicacid, methylester | Lauricacid, methylester | - | 0,001±0,000 | - | - | - | - | - | - | 0,028±0,015 |
| **9** | | 2,5-dimethyl-Benzaldehyde | | - | - | 0,003±0,000 | 0,010±0,001 | - | - | 0,004±0,001 | 0,017±0,001 | - |
| **10** | | di-Butyl-hydroxitoluene | BHT | 0,001±0,001 | 0,0005±0,0001 | 0,003±0,001 | 0,017±0,001 | 0,010±0,000 | 0,004±0,002 | 0,007±0,001 | 0,020±0,005 | - |
| **11** | | 1- Dodecanol | | 0,002±0,003 | 0,002±0,000 | 0,004±0,000 | 0,015±0,002 | 0,004±0,000 | 0,001±0,000 | 0,004±0,001 | - | - |
| **12** | | Dodecylacrylate | | 0,003±0,004 | 0,002±0,000 | 0,006±0,000 | 0,056±0,001 | 0,015±0,000 | 0,005±0,002 | 0,018±0,000 | - | - |
| **13** | C14:0 | Tetradecanoicacid, methylester | Myristicacid, methylester | 0,002±0,003 | 0,003±0,001 | - | 0,003±0,001 | 0,001±0,000 | 0,002±0,001 | 0,001±0,000 | 0,002±0,000 | 0,029±0,009 |
| **14** | C15:0 | Pentadecanoicacid, methylester | | - | - | - | - | - | - | - | - | 0,004±0,001 |
| **15** | | Nonanedioicacid, dimethylester | Azelaicacid, dimethylester | - | - | - | - | - | - | - | - | - |
| **16** | C16:0 | Hexadecanoicacid, methylester | Palmiticacid, methylester | 0,015±0,017 | 0,010±0,000 | 0,012±0,00 | 0,190±0,011 | 0,033±0,001 | 0,005±0,002 | 0,022±0,003 | 0,082±0,016 | 1,332±0,128 |
| **17** | C16:1 | (z) 9- Hexadecenoicacid, methylester | Palmitoleicacid, methylester | - | - | - | - | - | - | - | - | 0,032±0,008 |
| **18** | | 2,4-bis-(1,1-dimethyl ethyl) phenol | Antioxidante nº 33 | 0,008±0,010 | 0,010±0,000 | 0,006±0,000 | 0,018±0,001 | 0,012±0,000 | 0,004±0,002 | 0,013±0,001 | 0,030±0,004 | 0,010±0,001 |
| **19** | C17:0 | Heptadecanoicacid, methylester | | - | - | - | - | - | - | - | - | 0,013±0,002 |
| **20** | C17:1 | cis-10-Heptadecenoicacid, methylester | | - | - | - | - | - | - | - | - | 0,004±0,000 |
| **21** | C18:0 | Octadecanoicacid, methylester | Stearicacid, methylester | 0,009±0,011 | 0,008±0,000 | 0,006±0,001 | 0,067±0,003 | 0,021±0,001 | 0,004±0,002 | 0,013±0,001 | 0,045±0,005 | 0,687±0,005 |
| **22** | C18:1 | 9-Octadecenoic acid, methylester | Oleicacid, methylester | 0,007±0,009 | 0,001±0,000 | - | 0,065±0,001 | - | - | 0,001±0,000 | 0,006±0,001 | 1,858±0,019 |
| **23** | C18:1 | 9-Octadecenoic acid, methylester (isomer) | Oleicacid, methylester (isomer) | - | - | - | - | - | - | - | - | 0,063±0,020 |
| **24** | C18:2 | (z,z) 9,12-Octadecadienoic acid, methylester | Linoleicacid, methylester | 0,011±0,013 | - | - | 0,236±0,002 | - | - | 0,002±0,000 | - | 2,791±0,002 |
| **25** | | 3-mercapto-Propanoic acid, dodecylester | | - | - | - | - | - | - | - | - | - |
| **26** | C18:2 | 8,11-Octadecadienoic acid, methylester | | - | - | - | - | - | - | - | - | - |
| **27** | C18:3 | (z,z) 9,12, 15-Octadecatrienoic acid, methylester | Linolenic acid, methylester | 0,001±0,001 | - | - | 0,024±0,000 | - | - | 0,002±0,000 | - | 0,350±0,009 |
| **28** | | (3,5)-bis-(1,1-dimethylethyl)-4-hydroxy)benzenopropanoicacidmethylester | | 0,002±0,003 | - | 0,006±0,001 | - | 0,010±0,002 | 0,003±0,001 | 0,007±0,000 | - | 0,201±0,047 |
| **29** | | (z)-9- Octadecen-1-ol | Oleylalcohol | - | - | - | - | - | - | - | - | 0,019±0,007 |
| **30** | C20:0 | Eicosanoicacidmethyl, ester | Arachidicacid, methylester | - | - | - | - | - | - | - | - | - |
| **31** | C8:2 | 7,10- Octadecadienoicacid, methylester | | - | - | - | - | - | - | - | - | - |
| **32** | C8:2 | 9 cis, 11-trans-Octadecadienoic acid, methyl ester | | - | - | - | - | - | - | - | - | - |
| **33** | C20:1 | cis-11-Eicosenoicacid, methylester | | - | - | - | - | - | - | - | - | 0,010±0,002 |
| **34** | C20:4 | 5,8,11,14-Eicosatetraenoic acid, methylester | Arachidonicacid, methylester | - | - | - | - | - | - | - | - | 0,041±0,002 |
| **35** | | 9,10-hydroxy Octadecanoic, methylester | | - | - | - | - | - | - | - | - | - |
| **36** | C22:0 | Docosanoicacid, methylester | Behenicacid, methylester | - | - | - | - | - | - | - | - | 0,027±0,002 |
| **37** | | Hexadecanoic acid, 1- (hidroxymethyl)-1,2-ethenediyl ester | 1,2-di-Palmitin | - | - | - | - | - | - | - | - | 0158±0,79 |
| | | | **Total fatty acids** | 0,1±0,0 | 0,02±0,00 | 0,02±0,00 | 1±0 | 0,1±0,0 | 0,01±0,00 | 0,04±0,00 | 0,1±0,0 | 7,3±0,1 |

| Nr | C;D | Compounds | Common name | P06 | P07 | P09 | P10 | P12 | P15 | P16 | P17 | PC01 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2** | C6:0 | Hexanoic acid, 2- ethyl, methyl ester | | - | - | - | - | - | - | - | - | - |
| **3** | C8:0 | Octanoicacid, methylester | Caprylicacid, methylester | 0,049±0,002 | - | - | - | 0,003±0,002 | 0,002±0,000 | - | 0,0004±0,0001 | - |
| **4** | C6:2 | 2,4- Hexadienoicacid, methylester | | 0,016±0,001 | - | - | - | - | - | - | - | - |
| **5** | C10:0 | Decanoicacid, methylester | Capricacid, methylester | 0,086±0,041 | 0,002±0,000 | - | - | 0,023±0,017 | - | - | - | - |
| **6** | | Benzoicacid, methylester | | - | - | - | - | - | - | - | 0,001±0,000 | - |
| **7** | C10:1 | 4-Decenoic acid, methylester | | - | - | - | - | 0,002±0,001 | - | - | - | - |
| **8** | C12:0 | Dodecanoicacid, methylester | Lauricacid, methylester | 0,418±0,011 | 0,005±0,000 | - | - | 0,008±0,006 | - | - | 0,001±0,000 | - |
| **9** | | 2,5-dimethyl-Benzaldehyde | | - | - | - | - | - | 0,002±0,000 | 0,003±0,000 | - | - |
| **10** | | di-Butyl-hydroxitoluene | BHT | 0,013±0,003 | 0,007±0,001 | 0,040±0,006 | 0,037±0,004 | 0,004±0,002 | 0,007±0,001 | 0,005±0,000 | 0,007±0,001 | 0,001±0,000 |
| **11** | | 1- Dodecanol | | 0,011±0,000 | 0,004±0,001 | 0,012±0,002 | 0,024±0,001 | 0,005±0,003 | 0,005±0,000 | 0,003±0,000 | - | 0,001±0,000 |
| **12** | | Dodecylacrylate | | 0,064±0,009 | 0,001±0,000 | 0,042±0,007 | 0,026±0,001 | 0,007±0,004 | 0,036±0,004 | 0,011±0,001 | - | 0,003±0,002 |
| **13** | C14:0 | Tetradecanoicacid, methylester | Myristicacid, methylester | 0,259±0,002 | 0,035±0,007 | - | 0,002±0,000 | 0,006±0,003 | 0,001±0,000 | 0,001±0,000 | 0,009±0,002 | 0,001±0,001 |
| **14** | C15:0 | Pentadecanoicacid, methylester | | - | 0,001±0,000 | - | - | 0,001±0,000 | - | - | 0,001±0,000 | - |
| **15** | | Nonanedioicacid, dimethylester | Azelaicacid, dimethylester | - | 0,001±0,000 | 0,011±0,007 | - | 0,001±0,000 | - | - | - | - |
| **16** | C16:0 | Hexadecanoicacid, methylester | Palmiticacid, methylester | 0,351±0,019 | 0,355±0,063 | 0,073±0,011 | 0,057±0,013 | 0,295±0,071 | 0,111±0,007 | 0,021±0,000 | 0,070±0,010 | 0,005±0,002 |
| **17** | C16:1 | (z) 9- Hexadecenoicacid, methylester | Palmitoleicacid, methylester | - | 0,004±0,001 | - | - | 0,101±0,039 | - | - | - | - |
| **18** | | 2,4-bis-(1,1-dimethyl ethyl) phenol | Antioxidante nº 33 | - | 0,017±0,005 | 0,029±0,013 | 0,025±0,005 | 0,009±0,001 | 0,005±0,000 | 0,009±0,000 | 0,005±0,001 | 0,004±0,004 |
| **19** | C17:0 | Heptadecanoicacid, methylester | | - | - | - | - | 0,002±0,001 | - | - | 0,001±0,000 | - |
| **20** | C17:1 | cis-10-Heptadecenoicacid, methylester | | - | - | - | - | - | - | - | - | - |
| **21** | C18:0 | Octadecanoicacid, methylester | Stearicacid, methylester | 0,161±0,008 | 0,119±0,038 | 0,027±0,008 | 0,015±0,005 | 0,104±0,024 | 0,034±0,001 | 0,007±0,001 | 0,027±0,003 | 0,003±0,000 |
| **22** | C18:1 | 9-Octadecenoic acid, methylester | Oleicacid, methylester | 1,098±0,070 | 0,583±0,184 | 0,051±0,011 | 0,093±0,032 | 0,159±0,032 | 0,030±0,043 | 0,005±0,001 | 0,085±0,010 | 0,001±0,000 |
| **23** | C18:1 | 9-Octadecenoic acid, methylester (isomer) | Oleicacid, methylester (isomer) | 0,005±0,000 | 0,014±0,003 | - | - | 0,010±0,002 | 0,033±0,043 | - | - | - |
| **24** | C18:2 | (z,z) 9,12-Octadecadienoic acid, methylester | Linoleicacid, methylester | 0,016±0,002 | 1,071±0,378 | 0,055±0,008 | - | 0,106±0,030 | 0,145±0,001 | 0,003±0,000 | 0,026±0,003 | - |
| **25** | | 3-mercapto-Propanoic acid, dodecylester | | - | - | - | - | - | 0,006±0,001 | - | - | - |
| **26** | C18:2 | 8,11-Octadecadienoic acid, methylester | | 0,042±0,001 | - | - | - | - | - | - | - | - |
| **27** | C18:3 | (z,z) 9,12, 15-Octadecatrienoic acid, methylester | Linolenicacid, methylester | - | 0,044±0,021 | - | - | 0,030±0,008 | 0,012±0,000 | - | 0,003±0,000 | - |
| **28** | | (3,5)-bis-(1,1-dimethylethyl)-4-hydroxy)benzenopropanoicacidmethylester | | - | 0,018±0,009 | 0,054±0,011 | 0,154±0,021 | 0,011±0,002 | 0,007±0,000 | 0,004±0,000 | 0,012±0,001 | 0,002±0,005 |
| **29** | | (z)-9- Octadecen-1-ol | Oleylalcohol | 0,012±0,003 | - | - | - | 0,010±0,002 | - | - | 0,007±0,001 | - |
| **30** | C20:0 | Eicosanoicacidmethyl, ester | Arachidicacid, methylester | - | 0,012±0,005 | - | - | 0,006±0,002 | - | - | 0,002±0,001 | - |
| **31** | C8:2 | 7,10- Octadecadienoicacid, methylester | | - | 0,002±0,000 | - | - | - | - | - | - | - |
| **32** | C8:2 | 9 cis, 11-trans-Octadecadienoic acid, methyl ester | | 0,064±0,013 | - | - | - | - | - | - | - | - |
| **33** | C20:1 | cis-11-Eicosenoicacid, methylester | | - | 0,007±0,003 | - | - | - | - | - | - | - |
| **34** | C20:4 | 5,8,11,14-Eicosatetraenoic acid, methylester | Arachidonicacid, methylester | - | - | - | - | - | - | - | - | - |
| **35** | | 9,10-hydroxy Octadecanoic, methylester | | - | - | - | - | 0,010±0,003 | - | - | - | - |
| **36** | C22:0 | Docosanoicacid, methylester | Behenicacid, methylester | - | 0,002±0,001 | - | - | 0,01±0,00 | - | - | - | - |
| **37** | | Hexadecanoic acid, 1- (hidroxymethyl)-1,2-ethenediyl ester | 1,2-di-Palmitin | - | - | - | - | - | - | - | - | - |
| | | | **Total fatty acids** | 2,667±0,095 | 2,258±0,428 | 0,216±0,020 | 0,167±0,035 | 2,063±0,276 | 0,374±0,061 | 0,037±0,002 | 0,226±0,015 | 0,009±0,002 |

According to available information in the product labels (see index), we note that the amount of total fats of both of the products EP01 and P05 are respectively 0.5% and 6.5% despite that, the results shows that the total fatty acids contain in these products are respectively 1% and 7.3% .this diffence can be justificated with the type of methodology used into the analysis and it confirms that the quantities of fats written into the labels could not reflect the real amounts of the reality.

### 3.2 Results of free amino acids analysis

The identification and quantification of amino acids present in the supplements was performed by high performance chromatography equipped with a diode matrix detector and coupled to a mass detector (UPLC-MS-MS). For this analysis, only supplements identified as protein or containing high protein amounts were selected.

In the quantitative analysis, presented in Table 8, we can verify that for the evaluated supplements some amino acids are identified, with the samples C08, P10, P12 presenting the highest total amino acids with more than 43 mg/g. However, as it is too important for the bee to have access to a diverse set of amino acids, the larger amount does not necessarily reflect a good source of nutrients. In addition, as it is illustrated in the bibliography, the most balanced diets should contain ten essential amino acids to meet the nutritional requirements of bees, namely arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan and valine. Bees cannot synthesise them or even convert these amino acids to obtain them. [16]. In this sense, and according to the results obtained, P12 supplement appears as the richest and most balanced, followed by P05. Furthermore, C08, although containing an adequate proportion of most amino acids, the composition is excessively high in arginine, which may cause adverse effects.

**Table-8 Amino acid quantification (mg / g) performed by high performance liquid**

| | Samples | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Amino acids** | **C03** | **C05** | **C08** | **C09** | **C12** | **EP01** | **P03** | **P04** | **P05** | **P06** | **P07** | **P09** | **P10** | **P11** | **P12** | **P13** | **P14** | **P17** |
| Arg | - | - | 27,2±0,9 | 0,01±0,00 | - | 0,4±0,0 | - | - | 0,8±0,0 | - | 0,7±0,0 | 0,5±0,0 | 0,05±0,00 | 0,1±0,0 | 2,6±0,2 | 0,5±0,0 | 0,4±0,0 | - |
| Hist | - | - | - | - | - | 0,1±0,0 | - | - | 0,1±0,0 | - | - | - | 0,3±0,0 | 0,6±0,1 | 0,7±0,0 | 0,3±0,0 | - | - |
| Lys | - | - | 3,7±0,8 | - | - | 0,02±0,00 | - | - | 0,3±0,0 | 1,7±0,3 | 0,3±0,0 | - | 0,1±0,0 | - | 1,0±0,1 | 0,04±0,00 | - | - |
| Cys | - | - | - | - | - | - | - | - | - | - | - | - | 20,1±0,9 | - | 0,2±0,0 | 0,01±0,00 | - | - |
| Asn | 1,9±0,1 | - | - | - | - | - | - | - | 1,4±0,0 | - | 1,0±0,1 | - | 4,8±0,5 | 0,1±0,0 | 9,7±0,8 | - | - | - |
| Gln | - | - | 4,1±0,8 | - | - | - | - | - | 0,4±0,0 | 2,8±0,6 | 0,5±0,0 | - | 0,2±0,0 | - | 1,2±0,1 | - | - | - |
| Glu | - | - | 0,5±0,0 | - | - | - | 4±0 | - | 0,5±0,0 | 0,4±0,1 | 0,5±0,0 | 0,05±0,00 | - | - | 2,0±0,1 | 0,2±0,0 | 1,3±0,1 | - |
| Ser | - | - | 0,3±0,0 | - | - | - | - | - | 0,4±0,0 | - | | - | - | - | - | - | - | - |
| Thr | - | - | 0,5±0,1 | - | - | - | - | - | 0,2±0,0 | - | 0,1±0,0 | - | 0,1±0,0 | - | 1,0±0,2 | - | - | - |
| Asp | 0,1±0,0 | - | 2,6±0,4 | 0,04±0,00 | - | 0,1±0,0 | - | - | 0,1±0,0 | - | 0,1±0,0 | 0,1±0,0 | 0,2±0,0 | 0,1±0,0 | - | 0,1±0,0 | - | - |
| Val | - | - | - | - | - | - | - | - | 0,8±0,0 | - | | - | - | - | 2,7±0,4 | - | 4,9±0,3 | - |
| Met | 0,3±0,0 | - | - | 0,07±0,01 | - | 0,03±0,00 | - | - | 0,2±0,0 | 48,3±0,1 | 0,1±0,0 | 0,03±0,00 | 0,1±0,0 | 0,03±0,00 | 1,0±0,1 | 0,1±0,0 | - | - |
| Pro | 3,5±0,4 | - | 1,8±0,2 | - | - | 0,5±0,0 | - | - | 2,8±0,1 | - | 1,9±0,0 | - | 2,7±0,1 | - | 10,4±0,0 | 0,3±0,0 | 1,0±0,0 | - |
| Ile | 4,7±0,5 | - | 2,7±0,4 | - | - | 0,4±0,0 | - | - | 3,1±0,5 | - | 2,1±0,2 | - | 3,2±0,2 | - | 12,2±0,3 | 0,3±0,0 | 1,9±0,1 | - |
| Leu | 4,5±0,1 | - | 2,7±0,4 | - | - | 0,4±0,0 | - | - | 3,1±0,5 | - | 2,2±0,1 | - | 3,1±0,2 | - | 12,2±0,3 | 0,2±0,0 | 1,3±0,0 | - |
| Tyr | 0,1±0,0 | - | 4,3±0,5 | 0,05±0,00 | - | 0,1±0,0 | - | - | 2,9±0,3 | - | 0,7±0,0 | - | 0,4±0,0 | 0,5±0,0 | 2,2±0,1 | 0,3±0,0 | - | - |
| Phe | 5,4±0,2 | - | 7,4±0,0 | - | - | 0,9±0,0 | - | - | 2,3±0,1 | 0,3±0,0 | 2,7±0,0 | 0,04±0,00 | 3,8±0,6 | 3,4±0,1 | 24,0±1,7 | 0,5±0,0 | 3,5±0,1 | - |
| Trp | 2,2±0,1 | 0,04±0,00 | 6,3±0,4 | 0,3±0,0 | 0,06±0,01 | 3,5±0,2 | 1,2±0,3 | 0,1±0,0 | 1,4±0,1 | - | 2,7±0,0 | 0,5±0,0 | 3,9±0,2 | 2,4±0,1 | 14,0±0,2 | 3,1±0,1 | - | 0,04±0,00 |

Concerning leucine, isoleucine and valine, the amino acid requirements should be higher when comparing it with the other essential amino acids and the lack of one of these in the protein supplement can limit the development of the colony [14]. Altough, it was possible to detected high amounts of leucine, isoleucine and valine, the majority of the analysed products didn't contained these 3 amino acids at the same time, their presence was only detected in P05, P12 and P14 supplements.

Due to external factors like poor floral diversity, sometimes the proteins contained in the bee pollen are not sufficient for maintain bees' health. That's the reason, to obtain optimal nutrition, beekeepers balance their nutrient intake from complementary food sources to the protein supplements [13].

### 3.3 Results of minerals' analysis

The results for the minerals: potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) were obtained by flame ionization atomic absorption spectrophotometer, while the determination of the other minerals: manganese elements (Mn), Copper (Cu), and Cadmium (Cd), iron (Fe) and lead (Pb) were obtained by graphite chamber atomic absorption spectrophotometry, the results shown in Table 9 correspond to the analysis performed on the supplements.

As we can see, protein supplements are significantly richer in micronutrients. Generally, the most common elements are potassium, sodium, calcium and magnesium. Manganese and copper appears in some products in small quantities. Concerning elements associated with heavy metal contamination, cadmium, appears in only one food wich is P05, but in very small quantities.

The most obvious observations is the difference between the results obtained and the description provided on the product labels: firstly the identification of a higher number of minerals, secondely there's a disagreement with the values presented. In supplement E09 besides the indication of minerals zinc, potassium, sodium and manganese (undetected) it was found the presence of 23 mg of

magnesium. On the other hand, supplement P06, which according to the labeling information contained only sodium in the composition, showed high amounts of potassium, calcium and magnesium. However, excessive levels of sodium, and calcium have been shown to be toxic to honeybees [18], which may reduce the longevity of honeybees.

Once again, greater quality control of these products on the market becomes evident.

## Table-9 The results of minerals' analysis:

| Samples | K (mg/kg) | Na (mg/kg) | Ca (mg/kg) | Mg (mg/kg) | Mn (mg/kg) | Cu (mg/kg) | Fe (mg/Kg) | Cd (ppb) | Pb (mg/Kg) |
|---|---|---|---|---|---|---|---|---|---|
| **E01** | 18±1 | 3±1 | 85±2 | 14±2 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **E02** | 1111±14 | 15±2 | 95±2 | 7±0 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **E03** | 243±12 | 0,03±0,00 | 83±13 | 9±1 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **E04** | 22±0 | 8±0 | 146±4 | 8±1 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **E06** | 1152±7 | 21±2 | 35±2 | 7±2 | nd | nd | nd | nd | nd |
| **E07** | 61±0 | 12±1 | 89±2 | 11±0 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **E08** | 2,8±0,2 | 14±2 | 23,1±0,1 | 0,6±0,2 | nd | nd | nd | nd | nd |
| **E09** | 14±1 | 30,0±0,1 | 31±2 | 23±2 | nd | nd | nd | nd | nd |
| **E10** | 6533±0 | 399±0 | 1839±22 | 1131±38 | <13 | 39±5 | 165±6 | <0,7 | <1,4 |
| **E11** | 18227±50 | 1953±36 | 116±9 | 884±0 | 80±4 | <1,9 | <0,13 | <0,7 | <1,4 |
| **E12** | 7±1 | 17±3 | 77±3 | 10±1 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **E14** | 6±0 | 64±2 | 184±15 | 25±6 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **E15** | 42±1 | 47±3 | 106±11 | 12±2 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **E19** | 15160±44 | 1558±50 | 278±5 | 1665±38 | 8±0 | <1,9 | 14±0 | <0,7 | <1,4 |
| **EP01** | 4649±38 | 5997±1120 | 2208±9 | 67,9±0,0 | 0,66±0,03 | nd | nd | nd | nd |
| **EP02** | 66±3 | 99±0 | 104±14 | 17±2 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **P03** | 1486±1 | 106±1 | 198±2 | 74±3 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **P04** | 277±1 | 12±1 | 93±2 | 24±1 | 1±0 | <1,9 | 14±1 | <0,7 | <1,4 |
| **P05** | 3892±24 | 450±17 | 738±37 | 680±5 | 7±2 | 0,33±0,02 | nd | 0,03±0,00 | nd |
| **P06** | 3521±103 | 53670±4865 | 74125±2558 | 631±28 | 4±1 | 0,23±0,02 | nd | nd | nd |
| **P07** | 590±5 | 117±2 | 153±2 | 19±0 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **P09** | 21433±6 | 2±0 | 2665±22 | 2468±44 | 21±2 | 9±1 | 132±5 | <0,7 | <1,4 |
| **P10** | 18765±42 | 174±0 | 1101±10 | 1373±0 | 5±0 | 4±0 | 73±1 | <0,7 | <1,4 |
| **P11** | 11453±50 | 148±2 | 715±4 | 1045±5 | 3±0 | 2±0 | 50±0 | <0,7 | <1,4 |
| **P12** | 14569±90 | 308±13 | 956±1 | 1423±24 | 5±0 | 3±0 | 96±5 | <0,7 | <1,4 |
| **P15** | 19963±5 | 766±4 | 55859±30 | 4126±14 | 21±1 | 10±0 | 1135±19 | <0,7 | <1,4 |
| **P16** | 774±3 | 53±2 | 153±3 | 81±5 | 1±0 | <1,9 | 13±0 | <0,7 | <1,4 |
| **P17** | 19744±30 | 191±6 | 493±33 | 559±39 | 7±1 | 3±0 | 9±2 | <0,7 | <1,4 |
| **PC01** | 1023±2 | 60±0 | 274±2 | 4571±0 | <0,13 | <1,9 | 7±1 | <0,7 | <1,4 |
| **C02** | 873±0 | 14±3 | 96±8 | 7±1 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **C03** | 2388±1 | 2224±10 | 131±12 | 304±1 | 2±0 | 4±0 | 4±1 | <0,7 | <1,4 |
| **C04** | 5230±4 | 536±5 | 992±1 | 518±1 | 8±0 | 6±0 | 50±1 | <0,7 | <1,4 |
| **C05** | 5359±32 | 399±1 | 979±28 | 754±17 | 9±0 | 6±0 | 9±0 | <0,7 | <1,4 |
| **C06** | 778±1 | 35±5 | 95±5 | 22±1 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **C08** | 4672±28 | 251±10 | 1078±28 | 338±2 | 6±0 | <1,9 | 1±0 | <0,7 | <1,4 |
| **C09** | 938±30 | 37±2 | 7767±26 | 136±17 | 4±0 | <1,9 | 1±0 | <0,7 | <1,4 |
| **C11** | 1031±2 | 2547±31 | 28±1 | 49±1 | 37±1 | <1,9 | <0,8 | <0,7 | <1,4 |
| **C12** | 10162±13 | 81±0 | 5030±4 | 764±0 | 6±0 | 3±0 | 7±0 | <0,7 | <1,4 |
| **ES01** | 1162±0 | 34±1 | 155±1 | 58±1 | 1±0 | <1,9 | 1±0 | <0,7 | <1,4 |
| **ES02** | 666±4 | 15±0 | 213±2 | 33±3 | 0,2±0,0 | <1,9 | <0,8 | <0,7 | <1,4 |
| **ES03** | 782±3 | 104±0 | 83±2 | 18±0 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |
| **EC01** | 561±15 | 30±1 | 156±3 | 10±0 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **EC02** | 94±1 | 23±2 | 160±2 | 24±3 | <0,13 | <1,9 | 1±0 | <0,7 | <1,4 |
| **EC03** | 94±3 | 25±0 | 184±1 | 19±3 | <0,13 | <1,9 | <0,13 | <0,7 | <1,4 |

## CONCLUSION

Artificial supplementation of honeybee colonies become a major issue in beekeeping and now it is a common and growing pratice within Portuguese beekeepers. The key factor of ensuring bee's health is monitoring the nutritional status of the colony and saving bee population declines from nutritional shortages caused by several factors. In order to support beekeepers decision on the basis of an effective knowledge of the composition of the products found in the market, the fatty acids, aminoacids and minerals were evaluated.

This evaluation showed high quantities of fatty acids, amino acids and minerals in bee food supplements.

Bee foods presented high amounts of fatty acids which will, probably have a greater role in bee nutrition because sources with highest lipid contents are considered attractive to honeybees. Furthermore, the fatty acids detected may play the role of the inhiation of microorganisms.

Although these highest quantities may reflect a benific nutritional value of the products, it isn't the same case with the amino acids profile. Concerning these nutrients, the most balanced diets should contain ten essential aminoacids to satisfy the nutritional requirements of honeybees. According to the results obtained, the majority of food supplements didn't contain the totality of these essential building structures.

The mineral results showed high quantities of these compounds, which could be toxic to honeybees. Also, cadmium, generally associated with heavy metal contamination, appeared in one of these food supplements.

Generally, the analyses showed discrepancies between the results obtained and the description available and that makes clear the need for more quality control for these products destinated to honeybees and that may reduce their longitivity instead of being benific.

Finally, to continue this work, in vitro tests should associate it in order to

confirm the hypothisis and the impact of the products on honeybees.

# APPENDIX

## Table -10 Available information about food composition

| *CODE* | *Composition* | | | | | |
|---|---|---|---|---|---|---|
| | Carbohydrates | Water | Fats | Minerals | Proteins | Ashes |
| *E09* | Frutose (44%)<br>Glucose (42%)<br>Sacarose (13%) | 18% | - | Zn; Na; K; Mg | 0,03% | - |
| *P05* | - | 4,8% | 6,5% | Ca (0,5%), P (1%)<br>Na (0,5%)<br>K (0,5%) | 79% | - |
| *C08* | - | - | - | - | - | - |
| *C06* | 71% | 26,5% | 0,5% | - | 0,7% | 0,1% |
| *ES03* | 74,1% | 21,5% | - | - | 1,4% | - |
| *E01* | Sacarose; Glucose; Açúcares invertidos | - | - | - | - | - |
| *ES01* | Frutose<br>Glucose | - | - | - | - | - |
| *C11* | - | - | - | - | - | - |
| *E10* | Glucose (51%)<br>Frutose (42%)<br>Maltose (2,5%)<br>Triose (2%)<br>Sacarose (2%) | - | - | - | - | - |
| *PC01* | 36,3% | 41,7% | - | - | 8,5% | 2,5% |
| *EC01* | Frutose<br>Glucose | - | - | - | - | - |
| *ES02* | Frutose<br>Glucose | - | - | - | - | - |
| *P11* | Pres. | - | Pres. | - | Pres. | - |
| *EP02* | Frutose<br>Glucose | - | - | - | Pres. | - |
| *E19* | Frutose (38%)<br>Glucose (32%)<br>Sacarose (7%) | 21% | - | Ca; Mg; P; Na; K; Cl; Fe; S | - | - |
| *C03* | - | - | - | Pres. | Pres. | - |
| *E08* | Pres. | Pres. | - | - | - | - |
| *EC02* | - | - | - | - | - | - |
| *EC03* | 75% | 20% | - | - | - | - |
| *E02* | Frutose (60%)<br>Glucose (40%) | - | - | - | - | - |
| *C02* | - | - | - | - | Pres. | - |
| *C01* | - | - | - | - | Pres. | - |
| *E03* | Frutose<br>Glucose | - | Pres. | - | - | <0,01% |

| | | | | | | |
|---|---|---|---|---|---|---|
| *E06* | Pres. | - | - | - | - | - |
| *P03* | 93% | - | <0,25% | Ca <0,01%<br>P <0,01%<br>Na 57mg/kg | 1,26% | <0,5% |
| *P04* | 93,35% | - | <0,25% | Ca 45mg/kg<br>P <108mg/kg<br>Na 57mg/kg | 1,38% | <0,5% |
| *EP01* | 11% | - | 0,5% | Ca (0,3%)<br>P (11%)<br>Na (0,4%) | 45% | - |
| *P06* | - | 4,8% | 6,50% | Ca – 0,50%<br>P – 1%<br>Na – 0,5%<br>P – 0,5% | 79% | 4,6% |
| *P17* | - | - | - | - | - | - |
| *P07* | 10% | - | 3,9% | 4% | 36,4% | - |
| *E04* | 78% | - | - | - | - | - |
| *E11* | Frutose<br>Glucose<br>Sacarose | - | - | - | - | - |
| *P08* | - | - | - | - | - | - |
| *C04* | 13% | - | 7% | 4% | 61% | - |
| *C05* | 13% | - | 7% | 4% | 61% | - |
| *C07* | - | - | - | Sódio | Pres. | - |
| *P10* | - | - | - | Ca (1,2%)<br>Mg; F; K; Na;<br>Zn | - | Pres. |
| *P12* | - | - | - | Pres. | - | - |
| *E12* | Frutose<br>Glucose | - | - | - | - | - |
| *E07* | Pres. | - | - | - | - | - |
| *P09* | - | - | - | Ca; P; Na; Mn | - | Pres. |
| *C09* | Sacarose | - | - | - | - | - |
| *A1* | 23% | 77% | - | - | - | - |
| *C12* | Sacarose<br>Frutose | - | - | - | - | - |
| *P13* | - | - | - | Pres. | - | - |
| *P14* | - | - | - | - | Pres. | - |
| *C10* | Pres. | - | - | NaCl | - | - |
| *C13* | - | - | - | - | - | - |
| *P16* | - | - | - | - | - | - |
| *E15* | - | - | - | - | - | - |
| *E16* | - | - | - | - | - | - |
| *E14* | - | - | - | - | - | - |

**Note**: The information available for each of the products purchased is in most situations very limited, only with generic descriptions of impact on bees, without clear evidence of their composition, mode and dosage of application, storage conditions, among others. Table 9 describes all product composition information obtained from the descriptive labels, information available on the Internet, product sheets and / or contact with the supplier.

# REFERENCES


[1] Wiston M.L., The Biology of the Honey Bee, Harvard University Press, Massachusetts, 1991.

[2] JARA L., Cepero A., Garrido-Bailón, E., Martin-Hernández, R., Higes, M., De la Rua, P. Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee, Journal of Invertebrate Pathology, 2012, 110, 8-13.

[3] Jean-prost P.1987. Apiculture (Connaitre L'abeille- Conduire le rucher). Ed. 6e édition TEC et DOC. Lavoisier, Paris, 309-341.

[4] https://agdev.anr.udel.edu/maarec/honey-bee-biology/seasonal-cycles-of-activities-in-colonies/, accessed in 29 of January of 2019.

[5] Alphandéry R., La route du miel (Le Grand livre des abeilles et de l'apiculture), 17- 18.

[6] Zangirolami M.S., Evaluation of the composition, safety and the impact of food supplements on bees marketed in Portugal, Master thesis, Instituto Politécnico de Bragança, Portugal, 2018.

[7] Jingeaux, Eric, L'abeille, les produits de la ruche (L'apiculture), 2010, 5.

[8] Gall, D., Nakano O., NetoS.S., CarvalhoR.P.L., BaptistaG. C.de, Filho E.B., ParraJ.R.P., Zucchi R.A., Alves S.B.,VendramimJ.D., MarchiniL.C., LopesJ. R.S., Omoto,C. 2002. Entomologia Agrícola**.** Piracicaba: BibliotecadeCiênciasAgráriasLuiz de Queiroz.

[9] Barbonçan, M. 2006. La vie sociale de la colonie, Edited by Rstica,(second edition),Paris, 54-83.

[10] Suwannapong G., Benbow M.E., Nieh J.C. 2011.Biology of Thai Honeybees: Natural History and Threats. In: Bees: Biology, Threats and Colonies, Edited by Richard M. Florio, Chapter 1, 1-98.

[11] Chouhaine M. 2010. Contribution à l'étude de la biodiversité des Apoidae en Tunisie, Etude morpho métrique, moléculaire et éco physiologique de l'abeille Tunisienne *Apis mellifera intermissa* 23.

[12] Wright G.A., Nicolson S.W., Shafir S. 2018. Nutritional Physiology and Ecology of Honey Bees. Annual Review of Entomoly, 63, 327-344.

[13] Ellis A.M., Hayes JR G.W.2009. An evaluation of fresh versus fermented diets for honey bees ( *Apis mellifera* ), Journal of Apiculture research, 48,215-216.

[14] Belitz H.D.Grosh.W.1986.Food chemistry, 7,201,304,321.

[15] Vaudo A.D., Tooker J.F., Grozinger C.M., Patch H.M. 2015. Bee nutrition and floral resources restoration.Current opinion in Insect Science, 10, 133-141.

[16] Brodschneider R., Crailsheim K. 2010. Nutrition and health in honey bees.Apidologie, 41, 278-294.

[17] Roulston T.H., Cane J.H., Buchmann S.L. 2000.What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny? Ecological Monographs, 70, 617–643.

[18] Roulston T.H., Cane J.H. (2000) Pollen nutritional content and digestibility for animals, Plant Systematics and Evolution, 222, 187–209.

[19] De Groot A.P. (1953) Protein and amino acid requirements of the honeybee (*Apismellifera* L.), Physiologia Comparata et Oecologia, 3, 197–285.

[20] K. Bouhadjra, Etude de l'effet des antioxydants naturels et de synthèse sur la stabilité oxydative de l'huile d'olive viérge 2011, Université Mouloud Mammeri Tizi-Ouzou: Faculté des sciences, 94

[21] Herbert Jr. E.W. 1992. Honey Bee Nutrition. In The Hive and the Honey Bee, Edited by Graham, J.M., Dadant& Sons, Hamilton, Illinois, 197-224.

[22] Crailsheim K., Schneider L.H.W., Hrassnigg N.,Bühlmann G., Brosch U., Gmeinbauer R.,Schöffmann B. 1992. Pollen consumption and utilization in worker honeybees (Apis mellifera carnica): dependence on individual age and function, Journal of Insect Physiology, 38, 409–419.

[23] Rogers R.E .1995 . Choose carbohydrates carefully for your bees. American Bee Journal, 742.

[24] Pesante D.G, Rindener T.E.,Collins A.M., Boykin P.L.,Buco S.M.1992.Honey production in Venezuela:effects of feeding sugar syrup on colony weight gains by Africanized and European colonies.Apidologie, 23,545-552.

[25] Taber,S.1963.Why bees collect pollen .XIX Int.Apic.Congr.:675.

[26] Barker ,R.J,Y.Lehner 1974.Acceptance and sustenance value of naturally occurring sugars fed to newly emerged adult workers of honey bees (Apis mellifera L.),J.Exp.Zool.187,277-286.

[27] Fatty acids in pollen: a review of their importance for honey bees.

[28] A review of native wild bee nutrition health. Megan E. Leach, Frank Drummond, International journal of ecology, 2018.