

AMINO ACID AND VITAMIN CONTENT OF PROPOLIS COLLECTED BY NATIVE CAUCASIAN HONEYBEES

Nazife Eroglu^{1*}Senem Akkus²Mustafa Yaman²Baris Asci³Sibel Silici⁴¹ The Scientific and Technological Research Council of Turkey² The Scientific and Technological Research Council of Turkey, Marmara Research Center Food Institute³ Ardahan University, School of Health Science⁴ Erciyes University, Faculty of Agricultural Biotechnology

* corresponding author email: nazifeeroglu@hotmail.com

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Abstract

The polyphenol content of propolis has received a lot of attention due to the benign biological properties noted in the chemical composition studies. However, there are very limited studies about other chemical components found in trace amounts *in nature* which contribute to the therapeutic properties of propolis. The present study, therefore, investigated the amino acid and vitamin composition of propolis. Propolis harvested by 60 colonies of *Apis mellifera caucasica* belonged to local non-migratory beekeepers. The *A. m. caucasica* is known for its distinctive propolis collecting capability which native to the secluded Ardahan Province of Turkey. Vitamin (Thiamine, Riboflavin) combinations of propolis were determined using the HPLC (High-Performance Liquid Chromatography) fluorescent detector. An amino acid analysis was also performed with the UFLC (Ultra-Fast Liquid Chromatography) system consisting of binary pump and UV/VIS. Our findings record that the vitamin and amino acid content of propolis samples collected from three areas of different altitudes in the same region differed from each other. Vitamin B1 content and Vitamin B2 content ranged between 0.025-0.16 mg/100g, and 0.304-0.777mg/100g, respectively. A maximum amount of amino acid was reported as leucine, while a minimum amount of amino acid was seen as tryptophan in Ardahan propolis. Consequently, the vitamin and amino acid content of propolis, which derived from secondary plant metabolites of resin, varied depending on their geographical altitudes. Those vitamin and amino acids found in the propolis composition are believed to have beneficial therapeutic properties.

Keywords: amino acids, B group vitamins, caucasian honeybees, propolis

INTRODUCTION

All animals need essential amino acids (EAAs) to develop, reproduce, and for somatic maintenance. These amino acids are required for the purpose of enzyme production, peptide or amine signaling, tissue repair, and basic somatic functions, such as the immune system. Essential amino acids are provided when consuming proteins found in other animals or plants. The need for these compounds decreases over the lifespan of an animal (Millward et al., 1997; Tigreros 2013, van de Rest et al., 2013).

Throughout the larval stage of the honeybee, plants are a source of pollen and nectar-provided protein, and other essential nutrients (Westrich, 1990). Protein in the honeybee diet is reported to be important for development, reproduction, and longevity (Roulston & Cane, 2002). Honeybees were reported to prefer plants with a high amino acid content (Alm et al., 1990). The essential amino acids in pollen (arginine 11%, histidine 5%, isoleucine 14%, leucine 16%, lysine 11%, methionine 5%, phenylalanine 9%, threonine 11%, tryptophan 4%, and valine 14%) were determined for honeybees (De Groot,

1953). The amino acids found in propolis were assumed to originate from plants with resinous substance as well as saliva resulting from bee metabolism (Marcucci et al., 1996). On the other hands, vitamins are organic compounds needed for providing normal growth and development. Vitamins aid the general wellbeing of animals and insects. Vitamins have different chemical compositions and biological functions, and most importantly are used for numerous metabolic pathways and the synthesis of essential co-factors controlled by enzymes and coenzymes (Ball, 1994). Water-soluble vitamins (Vitamins B1, B2, B6, B12, folic acid, pantothenic acid, niacin, Biotin, and Vitamin C) are involved in the metabolism of fats, carbohydrates, and proteins, at different stages. Water-soluble vitamins are not stored in the body.

Propolis is recorded to have more than 300 compounds. The chemical constituents are changed greatly by environmental conditions, such as region, season, climate, flora, altitude. Honeybees also affect the constituents (Miguel & Antunes, 2011; Popova et al., 2010; Sforcin, 2007). The compounds identified in propolis resin originate from 3 sources: 1) plant exudate collected by bees, 2) secreted substances from bee metabolism, 3) and materials which are introduced during propolis elaboration (Ghisalberti, 1979; Marcucci et al., 1994). The chemical composition of propolis is identified as 50% resin and vegetable balsam (composed of flavonoids and related phenolic acids called the polyphenolic fraction), 30% waxes, 10% essential and aromatic oils, 5% pollen, and other organic compounds (Burdock, 1998; Popova et al., 2010). The "other organic compounds" also make up the rest of 5%. These other compounds contain proteins, amino acids, amines and amides of total 0.7% of all and trace amounts of carbohydrates, lactones, quinones, steroids, and vitamins. In early studies, vitamins B1, B2, B6, C, E, and mineral elements of silver, cesium, mercury, lanthanum, antimony, copper, manganese, iron, calcium, aluminum, vanadium, and silicon have all been identified in propolis samples from different botanical and geographical origins (Deblock-Bostyn, 1982; Debuyser,

1983; Dogan et al., 2006). Yet, there are very few studies addressing the amino acid contents of propolis (Greenaway et al., 1990; Gabrys et al., 1986; Moreira, 1986).

Our research aimed to determine the vitamin (B1 and B2) and amino acid contents of Ardahan propolis and evaluate the quantity compare with previous studies.

MATERIAL AND METHODS

Collection of propolis samples

Sixty samples of Turkish propolis were obtained from three selected apiaries in Ardahan, north-eastern Turkey. Ardahan Province is surrounded by high mountains in the northeastern border of Turkey to the Caucasus region of Eurasia. Ardahan Province is a naturally formed, isolated rural area far away from industrialized zones. Traditional non-migratory beekeeping is very common due to the geography and climate. All of the apiaries in this study are owned by stationary local beekeepers that never migrate. Beekeepers' location and geographical zone was determined by GPS (Magellan). Each apiary which had 20 strong (8-10 bee covered frames) colonies utilized plastic propolis traps on the top of the hive during the experiment. Samples were hand-collected between August-October 2014, and kept at a deep freeze (-25°C), and protected from light. The Ardahan region had a total of 3x20=60 colonies. Propolis samples for the extraction were randomly chosen in three locations to represent the Ardahan area. Sample collecting locations were recorded as Ardahan-Center in the village of Kartalpinar [(38T) 0310696 E 4558178 N], Ardahan-Center in the village of Sugoze [(38T) 0303427 E 4552596 N], and Ardahan-Posof in the village of Kumlukoz [(38T) 0315551 E 4604278 N]. To make a uniform sampling, 10 grams of raw propolis were used from each beehive and were distributed accordingly for the analyses. The altitude of each location was recorded for the highest zone of Kartalpinar (1835 m), Sugoze (1746 m), and Kumlukoz (1431 m).

Identification of honeybee samples

Caucasian honeybees were identified by Duzce University, Department of Biology using classic morphometric techniques which considered the right forewing venation and angles.

Chemicals

In this study, most of the analytes were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany) except HPLC grade methanol which was purchased from Merck (Darmstadt, Germany).

Analysis of vitamins

Tiamin (Vitamin B1) analyses

1g of homogenized propolis sample was inserted in a 100 ml flask containing 60 ml of 0.1 N HCl and the solution was autoclaved at 121°C for 30 min. After cooling down to 37°C or room temperature, it was pH adjusted to 4.5 with sodium acetate solution and the following was added: taka-diastase, clara-diastase, papain, alpha-amylase, and acid phosphatase enzyme. After shaking in a water bath at 45°C for 3 hours, the sample cooled to room temperature and the volume was completed to 100 ml with a 0.1 N hydrochloric acid solution. Then the solution was filtered through ordinary filter paper and centrifuged at 4500 rpm for 5 min. Twenty-five ml of solution was placed into a polyethylene tube, and after that 1.5 ml potassium ferricyanide (1%w/v) solution was added, and then the pH was adjusted to 7.0 with an ortho-phosphoric acid solution. The mobile phase included 1000ml of phosphate buffer and 250 ml of methanol mix and an adjustment to a pH of 7. The detector was FLD (Fluorescence detector). The wavelength covered excitation of 366 nm and emission of 445 nm. High-performance liquid chromatography (HPLC) column was Agilent Eclipse X08-C18, 5µm, 4.6x150 mm with a flow rate of 1 ml/min (AOAC, 2011; Eitenmiller et al., 2008; Esteve et al., 2001; Finglas et al., 1984).

Riboflavin (Vitamin B2) analyses

1g of propolis was placed in a 100 ml flask

containing 60 ml of 0.1 N HCl, and the solution was autoclaved at 121°C for 30 min. After cooling down to 37°C, the pH was adjusted to 4.5 with a sodium acetate solution, and after that mixture of acid phosphatase and taka-diastase enzyme solution was added. After shaking in a water bath at 45°C for 3 hours, the solution was boiled for 10 minutes. The cooled sample solution was brought to 100 ml with 0.1 N HCl and centrifuged at 4500 rpm for 5 min. The final solution was filtered through ordinary filter paper and passed through a 0.45 µm filter and injected onto the HPLC. The mobile phase differed from the Tiamin extraction and included 1000 ml of phosphate buffer and 250 ml of methanol mix and an adjustment to a pH of 2.5. The detector was a FLD (Fluorescence detector). The wavelength covered excitation of 445 nm and emission of 525 nm. The high-performance liquid chromatography column was Agilent Eclipse X08-C18, 5µm, 4.6x150 mm with a flow rate of 1 ml/min (AOAC, 2011; Eitenmiller et al., 2008; Esteve et al., 2001; Finglas et al., 1984). All vitamin analyses were run twice.

Amino acid analyses

The analysis method included 17 amino acids (aspartic acid, glutamic acid, serine, glycine, arginine, histidine, threonine, lysine, alanine, proline, leucine, isoleucine, tyrosine, phenyl alanine, valin, methionine, and tryptophan). The procedure firstly requires the alkaline hydrolysis of protein for tryptophan and an acid hydrolysis for others in sealed glass bottles under N₂ atmosphere. Following the protein hydrolysis, the method involved filtration, extraction with a mixture of acetonitril, methanol and triethylamine, pre-column derivatisation and detection of 16 amino acids (Asp, Glu, Ser, Gly, Arg, His, Thr, Lys, Ala, Pro, Leu, Ile, Tyr, Phe, Val, and Met). The solution being used for derivatisation included Edman reagent. After derivatisation, separation was done on reverse-phase analytical columns and detection and analyses was done with the UFLC (Ultra fast liquid chromatography) system which consisted of a binary pump and UV/VIS detector.

Following hydrolysis, the hydrolysates were

filtered and the pH values were adjusted with HCl solution. Separation and detection occurred on a reversed-phase analytical column of HPLC using a fluorescence detector within 10 minutes (Gheslaghi et al., 2008; Dimova 2003). Amino acid analyses were done by double run for a single propolis sample. Each sample was also injected twice so that a single colony was represented four times.

RESULTS

The qualitative and quantitative analyses of the propolis samples gathered from selected villages of three different altitudes were determined. A highest content of amino acid in these samples was found in the village of Sugoze, followed by the villages of Kartalpinar and Kumlukoz. The entire 17 amino acids were present in all of the studied samples, however, individual amounts of amino acids varied depending on the location

(Tab. 1). Leucine was the most abundant amino acid found at the Sugoze location. In this location, more than 300 mg/kg leucine and proline, accompanying more than 200 mg/kg alanine and valine amino acids, 100 mg/kg of aspartic and glutamic acids, serine, glycine, threonine, isoleucine, phenylalanine, and lysine, were detected. Other amino acids which made up less than 100 mg/kg were also detected. The propolis samples obtained from the village of Kartalpinar displayed over 100 mg/kg of leucine, proline, alanine, threonine, valine, lysine, glycine, and aspartic acids, and less amounts of other amino acids. In Kumlukoz, leucine surprisingly, was the only amino acid found above 100 mg/kg from among all the amino acids; all other amino acids were detected below this value. The only difference among propolis samples produced by the same honeybee (*A.m. caucasica* L.) subspecies from the same isolated region, was the altitude. Consequently, from the amino acids, leucine had the highest rate determined

Table 1
Amino acid composition of propolis samples from Ardahan, northeast Anatolia, collected by Caucasian honeybees (*Apis mellifera caucasica*)

Amino acids	Ardahan-Center-Sugoze (mg/100gr)	Ardahan-Center-Kartalpinar (mg/100gr)	Ardahan-Posof-Kumlukoz (mg/100gr)
	Mean±SD	Mean±SD	Mean±SD
Aspartic acid	135.6±0.4	103.9±0.9	71.2±2.2
Glutamic acid	140.4±5.1	94.9±1.4	57.0±0.7
Serine	162.6±1.1	83.5±1.2	60.7±0.4
Glycine	190.2±0.8	104.4±1.2	75.2±0.1
Histidine	57.8±1.1	47.8±3.1	37.9±0.6
Arginine	76.0±0.0	52.8±0.9	37.1±0.2
Threonine	198.0±0.9	118.5±0.4	70.4±0.6
Alanine	291.2±5.1	132.3±4.6	74.6±2.2
Proline	301.0±6.6	154.9±3.6	81.0±2.6
Tyrosine	99.4±0.4	63.8±1.5	45.9±0.2
Valine	229.5±1.1	116.1±0.4	82.4±0.3
Methionine	55.5±3.7	30.9±0.7	28.3±0.1
Isoleucine	172.8±0.9	94.3±0.2	58.7±0.0
Leucine	332.0±1.1	163.2±1.2	115.5±0.2
Phenylalanine	160.4±0.5	89.5±0.4	55.5±0.2
Lysine	126.5±1.2	111.8±0.9	71.7±0.4
Tryptophan	35.5±0.9	33.9±0.1	24.9±0.5
Total	2764.5	1596.4	1048.0

in all propolis samples, followed by proline and alanine. The B1 and the B2 vitamin content of the propolis samples ranged from 0.16 to 0.39 mg/100 g, and from 0.304 to 0.777 mg/100 g, respectively (Tab. 2). While the highest vitamin B1 content was found in the Kumlukoz samples. The village of Sugoze was recorded with the most vitamin B2 content.

of the many pollen species were determined even though the amino acids were in relatively small quantities (Micheu *et al.*, 2000). However, there was an absence of tryptophan in some pollen types reported in some studies (Auclair & Jamieson, 1948; Roulston & Cane, 2000), while other studies reported trace amounts of tryptophan (Weiner, 2010). In another study, a

Table 2

Vitamin structure based on Vitamin B1, and B2, of propolis samples from Ardahan, northeast Anatolia collected by Caucasian honeybees (*Apis mellifera caucasica*).

Region	Vitamin B1 (mg/100g)	Vitamin B2 (mg/100g)
Ardahan-Posof-Kumlukoz	0.16	0.426
	0.16	0.437
Ardahan-Center-Sugöze	0.025	0.746
	0.027	0.777
Ardahan-Center-Kartalpinar	0.038	0.305
	0.039	0.304

*Standard deviation of vitamin samples 0.1568

DISCUSSION

Throughout their larval stage, honeybees are fed primarily by pollen and nectar gathered from the plants. Pollen and nectar are the main source of protein and other essential nutrients (Westrich, 1990). Protein and vitamins found in their diet are crucial for the development, reproduction, and lifespan of honeybees (Roulston & Cane, 2002). Studies done on honeybee diets reported the importance of the diets, and that 10 amino acids are needed for honeybee development. The 10 needed amino acids are arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine, and valine (De Groot, 1953). It is well known that the vital functions of honeybees depend on protein and vitamin content received from pollen. The full spectrums of essential amino acids in some

comparison was done of *Taraxacum officinale* pollens compiled by the honey bees versus hand-picked pollens. Proline and valine were found the most in the the hand-collected pollen (Loper & Coohen, 1987). The quantitative differences in pollen protein lead to differences in the growth stimulation of the hypopharyngeal glands of worker bees. The proteins that contain a suitable amount and variety of amino acids are needed for normal growth and development of honeybees. It is clear that plant pollens do not have the same essential amino acids both qualitatively and quantitatively. Therefore, honeybees chose to collect pollen from a variety of plants instead of certain plant species and prefer some pollen less than other pollen. Honeybee preference of pollen collection is significant to provide enough nutrition for the growth and development of bees from different plant sources (Cook et al., 2003).

Propolis is not the primary source of amino acids and vitamins for honeybees. Recent studies reported that propolis is often contaminated with a small amount of pollen and plant leaf fragments (Zarakomska & Maciejewicz, 1992). The differences in the amino acid composition in the propolis samples were attributed by Marcucci (1996) to the variation of plant resources. He identified 11 amino acids. But, aspartic acid, glutamic acid, threonine, proline, valine, tyrosine, and histidine may be present in all samples. There were no differences in the percentage of the alanine, aspartic, glutamic acids, glycine, and histidine, despite the different botanical origins. These amino acid were recorded to emanate from the pollen sources (Marcucci, 1996). It was noted by Gabrys et al. (1986) that total propolis acid extraction contains more than 40% (w/w) amino acid concentration determined by gas-liquid chromatography (GC), whereas arginine and proline were found to be above 50% of the crude acid extract. Additionally, proline amino acid which is one of the compounds of propolis, was reported to contribute to this activity by promoting the production of collagen and elastin. In another study, propolis was reported to contain B1, B2, B6, C, and Vitamin E, with some enzymes like succinic dehydrogenase, glucose- 6-phosphatase, adenosine triphosphatas, and acid phosphatase (Tikhonov & Mamontov, 1987). It was reported by Sorkun *et al.* (2001) that there was more amino acid content in the propolis collected from Erzurum in the East Anatolia than that collected from the Trabzon and Gumushane regions in the Black Sea area, Turkey. Since, there are inadequate references to compare our findings on the vitamin content of propolis, there is very little to say to interpret the results. However, Vitamin B1 and B2 in our propolis samples are believed to originate from pollen sources.

De Groot (1953) reported that methionine, arginine, tryptophan, lysine, isoleucine, phenylalanine, histidine, valine, leucine, and threonine were essential amino acids for honey bees, while tyrosine, cysteine, serine, hydroxyproline, alanine, glycine, and proline were non-

essential. In addition, of the essential amino acids, leucine, isoleucine, and valine were required in the greatest amounts (as the most essential amino acids), tryptophan, methionine, and histidine in the lowest amounts (as the least essential amino acids), while threonine, phenylalanine, arginine, and lysine were required in amounts in-between these.

The nutritional needs of honeybees in a colony vary according to age and performed tasks. Adult worker bees, for instance, give more of a priority to carbohydrates in their diets than to EAA. For younger bees, the nutritional needs, especially of EAA, are greater. These needs may change by age and also by being a forager (Paoli et al., 2014). Hence, a preference for different types of pollen in the honeybee diet is inevitable (Standifer et al., 1977). At this point, even though it is considered as contaminating propolis, the amino acid composition may provide us with knowledge about the nutrients needed by the honeybee at different stages of their life. Marcucci (1996) evaluated both leaf fragments of dicotyledonous plants and resins of propolis to determine the amino acid structure. His examination revealed important differences of methionine, phenylalanine, and tyrosine. It was reported that these amino acids either were associated or involved bee metabolisms. Indeed, the three sources of amino acid content of propolis were cited as plants, pollen contamination, and bee metabolisms (Marcucci et al., 1996).

In our study, leucine showed the highest abundance rates compared to the other amino acids in the analysed samples. The function of leucine is important to build muscle structure, which is needed the most by honeybees. Due to the excessive flight and muscle activities during propolis collecting time (scraping, making pellets, loading and unloading), it is believed that this amino acid is much-needed. A preference for leucine-rich pollen sources may involve propolis production. In addition, it is accepted by many researchers that the vitamin and amino acid content of propolis has therapeutic properties.

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