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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

AMINO ACID COMPOSITION AND SOME PHYSICOCHEMICAL PARAMETERS OF MULTI-FLORAL HONEY FROM MOUNTAINOUS REGIONS OF KYRGYZSTAN

Kırgızistan'ın Dağlık Bölgelerinden Elde Edilen Polifloralı (Çok Çiçekli) Balın Amino Asit Bileşimi ve Bazı Fizikokimyasal Parametreleri

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ABSTRACT

Mountain animal food products are at the center of attention due to their intrinsic value and, as such, mountain beekeeping products deserve attention and effort for their valorisation. The work aimed at investigating the quality traits of mountain honey samples from Kyrgyzstan, giving particular emphasis on the amino acid profiles and their possible relationship with the other chemical-physical characteristics. The moisture content, acidity, pH, and diastase activity of honey samples were within the limits established by normative documents. The honey samples showed a higher diastase activity (26.34 – 77.9 Schade units), which demonstrates the high quality and superiority of mountain honey. The amino acid content of Kyrgyz honey was investigated for the first time. The major amino acids were proline (1553 mg/kg), followed by phenylalanine (805 mg/kg), lysine (349 mg/kg), and arginine (261 mg/kg). The sum of essential amino acids ranged from 675 to 4506 mg/kg and that of total amino acids from 1539 to 8958 mg/kg. Weak positive correlations were found between the altitude of the collection area and asparagine, glutamine, histidine, glycine, threonine, alanine, proline, valine, and total amino acids. The results form a basis for the establishment of quality standards for mountain honey.

Keywords: Honey production, Amino acid composition, HPLC analysis, Amylase Activity, Acidity

ÖZ

Dağ hayvanı gıda ürünleri, içsel değerleri nedeniyle ilgi odağındadır ve bu nedenle dağ arıcılık ürünleri, değerlenmeleri için dikkat ve çabayı hak etmektedir. Çalışma, Kırgızistan'dan alınan dağ balı örneklerinin kalite özelliklerini araştırmayı, özellikle amino asit profillerine ve bunların diğer kimyasal-fiziksel özelliklerle olası ilişkilerine vurgu yapmayı amaçladı. Bal örneklerinin nem içeriği, asitliği, pH'ı ve diastaz aktivitesi, normatif belgeler tarafından belirlenen sınırlar içindeydi. Bal örnekleri, dağ balının yüksek kalitesini ve üstünlüğünü gösteren daha yüksek bir diastaz aktivitesi (26.34 – 77.9 Schade birimi) göstermiştir. Kırgız balının amino asit içeriği ilk kez araştırıldı. Başlıca amino asitler prolin (1553 mg/kg), ardından fenilalanin (805 mg/kg), lisin (349 mg/kg) ve arginin (261 mg/kg) idi. Esansiyel amino asitlerin toplamı 675 ila 4506 mg/kg ve toplam amino asitlerin toplamı 1539 ila 8958 mg/kg arasındaydı. Toplama alanının yüksekliği ile asparagin, glutamin, histidin, glisin, treonin,

alanin, prolin, valin ve toplam amino asit arasında zayıf pozitif korelasyonlar bulundu. Sonuçlar, dağ balı için kalite standartlarının oluşturulması için bir temel oluşturmaktadır.

Anahtar Kelimeler: Bal üretimi, Amino asit bileşimi, HPLC Analizi, Amilaz aktivitesi, Asitlik

GENİŞLETİLMİŞ TÜRKÇE ÖZET

Amaç: Kırgızistan'da bal üretimi önde gelen bir ekonomik sektördür ve önemi uzun süredir hafife alınmaktadır. Kırgızistan'da 2013 yılında bal üretimi 1609 ton olup, bunun 195 tonu ihraç edilmiştir. Ancak 2019 yılında bal üretimi 2322 tona yükselmiştir (FAOSTAT, 2019). Kırgızistan'da üretilen bal çoğunlukla polifloraldir. Monomorfik veya monofloral balın üretilmesi zordur, çünkü arıcılık mevsimi boyunca aynı anda birkaç mellifeous bitkiler çiçek açar. Bu nedenle Kırgız balının adı genellikle botanik kaynağıyla değil, toplama alanıyla belirtilir. Kırgızistan'daki en popüler ve ünlü bal türleri Toktogul, Sary-Chelek, Kara-Shoro ve At-Bashy gibi dağlık bölgelerden toplanır. Kırgızistan'ın dağ balının fizikokimyasal özelliklerini ve amino asit değerini açıklayan bilimsel çalışmaların eksikliği göz önüne alınarak, bu çalışma Kırgız balının fizikokimyasal parametrelerini ve amino asit içeriğini belirlemeyi ve belgelemeyi amaçlamıştır. Bu da Kırgız balının doğal bir gıda kaynağı olarak daha ileride ticarileştirilmesi için gerekli olan kalite standartlarının oluşturulması için gereklidir. İkinci bir amaç, amino asit içeriğinin balın diğer kalite parametreleri ve toplanan alanın yüksekliği ile korelasyonlarını tespit etmektir.

Gereç ve yöntemler: Araştırma için Kırgızistan'ın yedi dağlı bölgesindeki arıcılardan doğrudan 15 polifloral bal örnekleri (her bölgeden 2 numune) alınmıştır: Suusamyr (SU), Sary-Chelek (SC), Toktogul (TO), Issyk-Kul (YK), Talas (TA), Chon-Kemin (CK) ve Kara-Shoro (KS) ve iki toplama sezonu: ilkbahar ve yaz başında – Mayıs, Haziran ve yazın – Temmuz, Ağustos, 2015. İncelenen tüm bal örnekleri, analize kadar buzdolabında 4°C sıcaklıkta saklanmıştır. Numunelerin analizi 2015-2016 yıllarında gerçekleştirilmiştir. Nem, toplam asitlik, pH ve diastaz aktivitesi gibi kalite parametrelerinin analizi, Avrupa Bal Komisyonu'nun Uyumlaştırılmış Yöntemlerine göre yapılmıştır. Amino asit içeriği Yüksek performanslı sıvı kromatografisi sisteminde tayin edilmiştir. Verilerin istatistiksel analizi, SPSS yazılımı, sürüm 16.0 kullanılarak analiz edilmiştir.

Bulgular ve tartışma: Kırgız balının nem, toplam asitlik ve pH gibi kalite parametreleri uluslararası bal standardına ve ayrıca asitliği Codex standardına ve diğer araştırmacıların bulgularına uygun olduğu tespit edilmiştir. Araştırılan bal örneklerinin pH'ı tipik bir 3.7-5.5 aralığı ile ortalama 4.4 olmuştur. Bal Kalitesi ve Uluslararası Düzenleyici Standartlara göre, diastaz aktivitesi 8 diastaz birimine eşit veya daha az olmamalıdır. İncelenen bal örneklerinin diastaz sayısı Schade birimlerinde 8,9±0,1 ile 77,9±5,1 arasında olmuştur. Kırgız balında 19 amino asit tespit edilmiştir. Ortalama amino asit değerlerine göre Kırgız balının başlıca amino asitleri prolin (1553 mg/kg), fenilalanin (905 mg/kg), lisin (349 mg/kg), arginin (261 mg/kg), tirozin (258 mg/kg) ve histidindir (251 mg/kg), son dört amino asit esansiyel amino asitlerdir. Örneklerin prolin içeriği, toplam amino asitlerin %20-50'sini oluşturduğu belirlenmiştir, bir sonraki en belirgin amino asit ise, 85 - 2969 mg/kg arasında olan fenilalanin esansiyel amino asidi olmuştur. Kırgız balındaki özel bir fark, esansiyel amino asit lizinin 242 mg/kg ila 661 mg/kg aralığında üçüncü ana amino asit olmasıdır. Aynı bölgeden fakat farklı mevsimlerden toplanan örneklerdeki toplam amino asit içeriği aynı değildir. Örneklerin yüksek amino asit içeriği ve yüksek değişkenliği, dağ manzarasının biyolojik çeşitliliği ile ilgili olabilir. Çalışmada pH, diastaz aktivitesi, nem içeriği, asit içeriği ve amino asit içeriği arasındaki korelasyon araştırılmıştır. Amino asit içeriği ile pH ($r = 0.284$, $p = 0.585$) ve amino asit içeriği ile diastaz sayısı ($r = -0.074$, $p = 0.890$), amino asit içeriği ile asitlik ($r = -0.429$, $p = 0.05$) arasında istatistiksel olarak anlamlı bir ilişki gözlenmemiştir. Bununla birlikte, balın amino asit bileşimi, balın nem içeriği ile pozitif bir korelasyonda ($r = 0.905$, $p = 0.05$) olmuştur. Dağ balı ile ilgili sınırlı literatüre katkıda bulunmak için dağ balının antioksidan aktiviteleri ve bunların amino asitlerle ve ayrıca toplam polifenol içeriği ile korelasyonu hakkında daha ileri çalışmalara ihtiyaç vardır.

INTRODUCTION

Honey is a multi-component natural food, characterized as a supersaturated carbohydrate solution with high viscosity (Smanalieva and Senge 2009). Currently, about 200 components have been found in honey (Alvarez-Suarez et al. 2014). The

sensory and physical properties (colour, flavour), and chemical composition of honey depends on additional factors, such as the botanical origin and the regional and climatic conditions of the area in which it is collected (Lazaridou et al. 2004). Though the major constituents of honey are carbohydrates and water, honey also contains amino acids (AA), phenolic compounds, vitamins, minerals and enzymes. Floral honey contains about 0.1 – 1.6% protein (Chua et al. 2015), while in honeydew honey this quantity is about 3.0% and comes from animal or vegetal (e.g. pollen) sources. The amount of AA is about 1%, and proline is the major amino acid, comprising 50–85% of the total AA content (Anklam 1998, Hermosín et al. 2003). Therefore, the use of proline as an indicator of honey ripeness and naturalness is suggested (Ohe et al. 2000). Normal honey has an average proline content of 200 mg/kg; a value below 180 mg/kg indicates sugar adulteration (Bogdanov et al. 1997; Hermosín et al. 2003; Ampuero et al. 2004). However, naturally, the amount of proline in acacia honey is low (120 mg/kg) (Akgün et al. 2021). Besides proline, honey contains 26 AA, and their amount depends on the origin of the honey (nectar or honeydew), therefore the AA profiles of honey samples could indicate their botanical origin (Anklam 1998, Cotte et al. 2004). According to Chua et al. (2015) honey proteins showed antioxidant activities in the form of free radical scavenging and ferric reducing power. Honey contains many enzymes, and their activity is the basis for assessing honey quality. Diastase (α- and β-amylase) is the most important enzyme that enriches the nutritional and therapeutic function of honey (White and Rudyj 1978, Huang et al. 2019).

In Kyrgyzstan, honey production is a prominent economic sector, and its importance has long been underestimated. The volume of honey production in Kyrgyzstan in 2013 was 1609 tons, of which 195 tons were exported. In 2019, the production of honey increased to 2322 tons (FAOSTAT, 2019). The natural conditions in Kyrgyzstan greatly favour honey production: 90% of the territory of the republic is mountains and the mountain flora is diverse and rich, including 3,500 flowering plants (Fig. 1). There are more than 300 melliferous plants that produce nectar and pollen (Smanalieva 2008). According to Verma (1990), mountain honey produced from diverse melliferous flora is considered better in quality than honey from lowland areas, as a result of which it fetches a higher price.

Honey produced in Kyrgyzstan is mainly polyfloral. Monomorphic or monofloral honey is difficult to produce, as several powerful melliferous plants bloom simultaneously during the beekeeping season. Therefore, the name of Kyrgyz honey is usually indicated by the collection area and not by its botanical source. The most popular and famous kinds of honey in Kyrgyzstan are collected from mountainous regions such as Toktogul, Sary-Chelek, Kara-Shoro, and At-Bashy (Kadyrova and Smanalieva 2017). According to a palynological investigation, Toktogul honey has pollen of such herbs as blueweed (*Echium vulgare*), sainfoin (*Onobrychis* sp.) chervil (*Anthriscus sylvestris*), family *Brassicaceae*, mint (*Mentha* sp.), oregano (*Origanum* sp.), St. John's wort (*Hypericum* sp.), thyme (*Thymus*), sweet clover (*Melilotus officinalis*), dandelion (*Taraxacum*) and many other mountain plants (Smanalieva 2008).

Figure 1: Uzgen district, the flowering plants in June

Sary-Chelek is a nature reserve in Jalal-Abad province in western Kyrgyzstan. The main herbs that grow in this area are St. John's wort (*Hypericum* sp.), wormwood (*Artemisia* sp.), thyme (*Thymus*), blueweed (*Echium vulgare*), blackroot (*Cynoglossum* sp.), members of the family *Brassicaceae*, mint (*Mentha* sp.) and sainfoin (*Onobrychis* sp.). The honey collected in Kara-Shoro contains pollen of thyme (*Thymus*), St. John's wort (*Hypericum* sp.), sainfoin (*Onobrychis* sp.), members of the cruciferous family (*Brassicaceae*), black root (*Cynoglossum* sp.), oregano (*Origanum* sp.), cornflower (*Centaurea* sp.), wormwood (*Artemisia* sp.), bedstraw (*Gallium*) and saw-wort (*Serratula* sp.). In the At-Bashy region, sainfoin (*Onobrychis* sp.) is a main

perennial melliferous plant. There are eight species of this unique plant in Kyrgyzstan. Sainfoin honey is light coloured with a very delicate and unobtrusive taste and a pleasant aroma (Smanalieva 2008).

Given the lack of scientific studies describing the physicochemical attributes and amino acid value of mountain honey from Kyrgyzstan, this study aimed to determine and document the physicochemical parameters and amino acid content of Kyrgyz honey for the establishment of quality standards for Kyrgyz honey, which are essential for its further commercialization as a natural food source. A second aim was to test for correlations of amino acid content with other quality parameters of honey and altitude of the collection area.

MATERIALS AND METHODS

Honey samples

For the investigation, 15 polyfloral honey samples were obtained directly from beekeepers in seven mountain regions of Kyrgyzstan: Suusamyr (SU), Sary-Chelek (SC), Toktogul (TO), Issyk-Kul (YK), Talas (TA), Chon-Kemin (CK) and Kara-Shoro (KS), Karkyra (KA) and two collection seasons: in early summer – May, June; and summer – July, August 2015. All investigated honey samples were stored in a refrigerator at a temperature of 4 °C until the analysis. Analysis of the samples was carried out in 2015-2016.

Physicochemical measurements

Analyses of quality parameters such as moisture, total acidity, pH, and diastase activity were conducted according to the Harmonized Methods of the European Honey Commission (Bogdanov et al. 1997). The refractive index of honey samples was measured using a refractometer (Reichert Abbe Mark II Plus Refractometer, Reichert, Inc., NY, USA) at 20°C. The corresponding moisture content (%) was calculated using the relationship between refractive index and water content. The pH was determined in a 10% (w/w) solution of honey in distilled water by means of a pH meter (Mettler Toledo, Gießen, Germany). The free acid content was measured in a 10% (w/w) honey solution by acid-base titration with 0.1 M NaOH up to pH 8.1 (Mettler Toledo, Gießen, Germany) and the results were expressed as the milliequivalents per kg honey (meq/kg) (Bogdanov et al. 1997).

The determination of diastase (α-amylase) activity was performed using a spectrophotometer (Specord 50, Analytic Jena, Germany) and expressed as Diastase number (DN) in Schade units. DN is defined as the amount of enzyme that will convert 0.01 g of starch to the prescribed endpoint in 1 h at 40 °C under the conditions of the test (Bogdanov et al. 1997).

Determination of amino acid composition

15 honey samples were chosen (2 samples from each region) for the determination of amino acid composition. The 19 AA standards (aspartic acid, glutamic acid, serine, histidine, glycine, threonine, arginine, alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, tryptophan, phenylalanine, ornithine, and lysine) were obtained from Merck (Darmstadt, Germany).

Sample preparation, for further determination by High-Performance Liquid Chromatography (HPLC), was conducted following the methodology proposed by (Bouseta et al. 1992) with some modifications by (Mazhitova and Kulmyrzaev 2016). Briefly, 0.2 g of honey dissolved in approx. 6 ml of high purity water and mixed thoroughly. The pH was adjusted to 3.2 with 0.1 N HCl. After isolation, the final sample volume was adjusted to 10 mL, and the sample was refrigerated before derivatization. The derivatization procedure and quantification by HPLC were conducted according to Hermosín et al. (2003); 0.5 mL of the solution of isolated AA was mixed with 3 µL of diethyl ethoxymethylenemalonate, 0.75 mL of methanol and 1.747 mL of borate buffer at pH 9.0 (1 M) and placed in a 10 mL tube with screw cap. The tube was closed and briefly shaken. After that, the mixture was placed in an ultrasound bath for 30 min at room temperature. Before injection (20 µL) into the HPLC (Agilent Technologies 1200, US), derivatized samples were filtered through a 0.45-µm microfilter. Chromatographic separation was carried out in a C18 column (4.6 mm × 250 mm × 5 µm) (G1316A, Agilent Technologies 1200, US) at 16 °C. Mobile phase consisting of acetonitrile (A) and 0.1 M acetate buffer (B) at pH 6.0 was used with following gradient conditions: 6% (A) at 0 min; 16% (A) at 13 min; 18% (A) at 13.5 min; 18% (A) at 17 min; 22% (A) at 20 min and 32% (A) at 32 min. A diode array detector at 280 nm was used. Identification was made using the retention time obtained from pure compounds and quantification was carried out according to the external standard method with 0.01, 0.5, 1.0, 2.0, 3.0, and 4 µg/mL concentration levels.

Statistical analysis

Data were analysed by ANOVA using SPSS software, version 16.0 (SPSS Inc.Chicago, IL). A two-step cluster analysis of individual AA by altitude was carried out. According to the two-step cluster analysis of individual AA by altitude, the samples can be divided into three groups: 1. Low and rough mountain honey; 2. Rugged mountain honey and 3. High mountain honey. A comparative analysis of individual AA and total AA between samples was performed using Duncan's test at a significance level of $p \leq 0.01$ and $p \leq 0.05$. Pearson's Correlation Coefficient Analysis was carried out to determine the association between the total amino acid content and altitude, also the physicochemical characteristics of honey.

RESULTS

Quality parameters of Kyrgyz honey

The results of analysis of quality parameters such as moisture, total acidity, and pH are provided in Table 1. The quality parameters of Kyrgyz honey were in compliance with the international honey standard (Codex Alimentarius 2001). The average moisture content was 17.26%, with a min value of 14.7% (KA21) and a max value of 23.7% (KS12).

Table 1. Some physicochemical parameters of the honey samples and the altitude of the collection area

	№ of sample	GPS Coordinates	Altitude	Moisture content, %	Acidity, meq/kg	pH	Diastase Number, Schade unit
1.	CH11	Latitude: 42.69 North Longitude: 75.32 East	1225	18.5±0.2	25.0±0.5	5.5±0.0	18.9±0.5
2.	TO11	Latitude: 41.88 North Longitude: 73.38 East	1901	18.0±0.5	18.2±0.4	4.6±0.0	28.8±2.4
3.	TO25	Latitude: 41.88 North Longitude: 73.38 East	1901	16.4±0.6	25.3±0.7	4.5±0.1	77.9±5.1
4.	TA24	Latitude: 40.33 North Longitude: 73.38 East	1372	15.9±0.1	13.6±0.3	4.0±0.1	18.8±1.9
5.	KS11	Latitude: 40.33 North Longitude: 73.38 East	1546	19.5±0.7	20.3±0.5	4.9±0.0	20.0±8.5
6.	KS12	Latitude: 40.33 North Longitude: 73.38 East	1546	23.7±0.2	16.3±0.6	4.6±0.0	33.4±2.8
7.	YK11	Latitude: 42.66 North Longitude: 78.62 East	1874	20.0±0.3	17.1±0.8	4.1±0.0	8.9±0.1
8.	SC22	Latitude: 41.89 North Longitude: 71.95 East	2218	20.3±0.8	20.3±0.5	4.8±0.0	17.0±7.1
9.	SC25	Latitude: 41.89 North Longitude: 71.95 East	2218	15.9±0.3	32.1±0.5	4.5±0.3	38.9±2.5
10.	SU21	Latitude: 42.18 North Longitude: 73.96 East	2132	15.8±0.5	17.2±0.3	4.1±0.0	10.5±0.5
11.	SU24	Latitude: 42.18 North Longitude: 73.96 East	2132	16.2±0.5	18.5±0.6	4.3±0.0	37.2±7.7
12.	CK21	Latitude: 42.76 North Longitude: 76.26 East	2000	15.9±0.2	25.5±0.6	4.4±0.1	32.9±9.8
13.	AB23	Latitude: 41.19 North Longitude: 75.86 East	2121	16.4±0.7	17.2±1.5	3.7±0.1	26.3±5.2
14.	AB24	Latitude: 41.19 North Longitude: 75.86 East	2121	15.3±0.5	25.2±0.2	4.5±0.1	52.9±2.2
15.	KA21	Latitude: 42.75 North Longitude: 79.15 East	2029	14.7±0.1	20.0±0.7	4.2±0.0	38.6±2.3

Means (n = 3),* Standard deviations SD ≤0.5, ** SD ≤0.05; AB-At-Bashy, CH- Chui, CK-Chon-Kemin, KS-Kara-Shoro, SC-Sary-Chelek, SU-Suusamyr, TA-Talas, TO-Tokotogul, YK-Yssyk-Köl, KA-Karkyra.

The amino acid profile of honey samples

Nineteen AA were determined in Kyrgyz honey. According to the two-step cluster analysis of individual AA by altitude, the samples from cited 7 Kyrgyz regions belonging to one of the 3 altimetric belts identified: 1. Low and rough mountain honey, 2. Rugged mountain honey and 3. High mountain honey. The mean values of these three groups were compared with one-way ANOVA (Table 2, 3, 4). The average AA content of Kyrgyz honey is depicted in Fig. 2

Table 2. Amino acids of honey samples from rough mountain region (mg/kg)

Amino acid	CH11		TA24		Min	Max
Asp	44.18^{k}	0.23	85.99^{e}	2.46	44.18	85.99
Glu	110.75^{f}	0.27	133.23^{d}	49.56	110.75	133.23
Ser	51.90^{j}	0.03	60.48^{f}	18.96	51.90	60.48
His	178.07^{e}	0.40	82.79^{e}	48.29	82.79	178.07
Gly	24.23^{m}	0.17	21.34h^{ij}	0.48	21.34	24.23
Thr	35.91^{l}	0.26	17.82^{ij}	0.05	17.82	35.91
Arg	242.43^{d}	0.44	141.78^{d}	0.16	141.78	242.43
Ala	59.62^{i}	0.16	38.70^{gh}	0.39	38.70	59.62
Pro	1128.02^{a}	11.76	656.44^{a}	6.93	656.44	1128.02
Tyr	82.82^{g}	0.39	53.08^{fg}	0.34	53.08	82.82
Val	65.68^{h}	7.46	31.58^{hi}	0.91	31.58	65.68
Met	3.48^{o}	0.42	2.96^{j}	0.08	2.96	3.48
Cys	ND		ND			
Ile	23.17^{m}	0.41	16.63^{ij}	2.46	16.63	23.17
Leu	18.63^{m}	0.61	16.44^{ij}	0.03	16.44	18.63
Trp	ND		14.89^{ij}	1.42	0.00	14.89
Phe	328.25^{c}	0.63	201.22^{c}	0.11	201.22	328.25
Orn	10.44^{n}	0.40	3.57^{j}	1.04	3.57	10.44
Lys	377.50^{b}	16.88	259.29^{b}	2.94	259.29	377.50
Sum of EAA	**935.43**		**613.91**		**613.91**	**935.43**
Total	**2785.08**		**1838.23**		**1838.23**	**2785.08**

Means (n = 3) ± standard deviations within a column with small superscripts differ significantly ($p < 0.05$). Asp – aspartic acid, Glu – glutamic acid, Ser – serine, His – histidine, Gly – glycine, Thr – threonine, Arg – arginine, Ala – alanine, Pro – proline, Tyr – tyrosine, Val – valine, Met – methionine, Cys – cysteine, Ile – isoleucine, Leu – leucine, Trp – tryptophan, Phe – phenylalanine, Orn – ornithine, Lys – lysine; ND – not detected; EAA- Essential amino acids.

Table 3. Amino acids of honey samples from rugged mountain region (mg/kg)

Amino acid	YK11		TO11		TO25		Min	Max
Asp	51.84^{hC}	3.64	89.07^{jA}	4.47	79.42^{nB}	0.13	51.84	89.07
Glu	107.34^{fC}	3.65	182.74^{gB}	0.70	252.95^{gA}	0.19	107.34	252.95
Ser	55.14^{hB}	0.65	101.22^{iA}	2.00	101.74^{lA}	0.49	55.14	101.74
His	187.90^{dC}	1.42	306.26^{fB}	4.44	360.91^{fA}	1.87	187.90	360.91
Gly	30.68^{iC}	0.47	57.70^{mB}	1.52	81.26^{nA}	0.11	30.68	81.26
Thr	31.58^{iC}	0.35	63.69^{lB}	1.50	89.21^{mA}	0.21	31.58	89.21
Arg	286.46^{cC}	0.60	349.52^{dB}	1.46	380.70^{eA}	0.30	286.46	380.70
Ala	53.14^{hC}	0.04	122.95^{hB}	0.58	210.39^{iA}	0.72	53.14	210.39
Pro	1474.90^{aB}	11.58	1295.52^{bC}	10.59	2085.53^{aA}	13.60	1295.52	2085.53
Tyr	81.11^{gC}	1.57	467.25^{cA}	1.47	452.07^{cB}	0.44	81.11	467.25
Val	140.50^{eB}	31.05	124.81^{hB}	3.97	169.07^{jA}	0.65	124.81	169.07
Met	1.15^{kC}	0.27	8.75^{oA}	0.34	6.44^{pB}	0.58	1.15	8.75
Cys	ND		ND		ND		-	-
Ile	24.49^{iC}	0.05	77.95^{kB}	8.10	136.86^{kA}	0.32	24.49	136.86
Leu	24.56^{iC}	0.42	86.14^{jB}	5.32	234.79^{hA}	0.34	24.56	234.79
Trp	10.46^{iC}	0.33	24.97^{nB}	5.77	46.36^{oA}	0.33	10.46	46.36
Phe	283.77^{cC}	6.01	1952.20^{aA}	13.48	1495.14^{bB}	0.01	283.77	1952.20
Orn	13.19^{jA}	0.04	11.55^{oB}	0.05	6.38^{pC}	0.13	6.38	13.19
Lys	440.42^{bA}	6.41	337.50^{eC}	0.50	405.97^{dB}	0.49	337.50	440.42
Sum of EAA	**1038.04^{C}**		**3143.25^{A}**		**3035.91^{B}**		**1038.04**	**3143.25**
Total	**3298.62^{C}**		**5659.79^{B}**		**6595.19^{A}**		**3298.62**	**6595.19**

Means (n=3)±standard deviations within a column with small superscripts, within a row with capital superscripts differ significantly ($p<0.05$). Asp–aspartic acid, Glu–glutamic acid, Ser–serine, His–histidine, Gly–glycine, Thr–threonine, Arg–arginine, Ala–alanine, Pro–proline, Tyr–tyrosine, Val–valine, Met–methionine, Cys–cysteine, Ile–isoleucine, Leu–leucine, Trp–tryptophan, Phe–phenylalanine, Orn–ornithine, Lys–lysine; ND–not detected; EAA-Essential amino acids.

Table 4. Amino acids of honey samples from high mountain region (mg/kg)

Amino acid	SU21	SU24	CK21	AB23	AB24	KA21
Asp	51.11^{gG}	49.64^{jG}	61.62^{iF}	82.61^{iE}	43.50^{hH}	83.97^{iE}
Glu	78.53^{fI}	87.65^{gH}	108.60^{fG}	156.61^{gE}	65.62^{fJ}	151.08^{gF}
Ser	48.61^{gG}	59.56^{iF}	82.15^{gE}	87.14^{hD}	48.72^{gG}	58.69^{jF}
His	167.03^{eG}	182.81^{eF}	179.51^{eF}	209.28^{fE}	117.83^{dH}	171.34^{fG}
Gly	21.77^{iG}	25.49^{lF}	36.79^{mD}	32.80^{mE}	14.67^{lH}	21.64^{nG}
Thr	18.19^{ijI}	76.51^{hC}	48.23^{kF}	44.19^{lG}	15.12^{lJ}	36.90^{lmH}
Arg	161.39^{eI}	164.76^{fH}	233.90^{dD}	218.19^{eE}	126.24^{cJ}	194.95^{eG}
Ala	48.29^{gH}	48.72^{jH}	77.55^{hE}	81.74^{iD}	24.54^{jI}	61.79^{jG}
Pro	739.65^{aI}	919.10^{aH}	1439.80^{bD}	1264.17^{bF}	650.29^{aJ}	1098.87^{bG}
Tyr	315.56^{bD}	311.78^{bE}	272.25^{cF}	530.47^{cA}	28.02^{iJ}	432.48^{cB}
Val	36.12^{hG}	44.59^{kFG}	79.67^{ghE}	58.67^{kF}	18.74^{kH}	107.14^{hD}
Met	1.34^{lGH}	1.77^{oG}	10.33^{oD}	8.49^{oE}	2.65^{oG}	ND
Cys	ND	ND	ND	27.27^{nB}	ND	ND
Ile	19.53^{ijH}	21.23^{mG}	53.43^{jE}	58.02^{kD}	11.02^{mI}	43.41^{klF}
Leu	39.89^{hH}	42.66^{kG}	51.34^{jkF}	66.73^{jD}	1.43^{oJ}	28.94^{mnI}
Trp	12.29^{jkF}	13.04^{nF}	42.08^{lC}	57.09^{kA}	7.62^{nG}	52.99^{jkB}
Phe	204.49^{dH}	201.70^{dH}	1601.27^{aC}	2971.61^{aA}	86.64^{eI}	1658.31^{aB}
Orn	8.14^{klEF}	11.36^{nC}	14.90^{nB}	8.19^{oEF}	8.67^{mnE}	4.03^{oG}
Lys	242.31^{cG}	300.08^{cC}	271.52^{cEF}	283.74^{dDE}	267.99^{bF}	275.29^{dDFE}
Sum of EAA	**889.72^{I}**	**1013.36^{G}**	**2430.11^{D}**	**4106.29^{A}**	**439.24^{J}**	**2635.45^{C}**
Total	**2214.23^{I}**	**2562.44^{H}**	**4664.93^{E}**	**6247.01^{C}**	**1539.32^{J}**	**4481.82^{F}**

Table 4. Amino acids of honey samples from high mountain region (mg/kg) (continuation)

Amino acid	SC22	SC25	KS11	KS12	Min	Max
Asp	231.33^{hA}	106.72^{gD}	126.79^{kC}	163.77^{kB}	43.50	231.33
Glu	404.04^{eB}	162.08^{fD}	308.65^{fC}	589.89^{dA}	65.62	589.89
Ser	175.57^{jB}	81.39^{hE}	152.09^{jC}	206.55^{iA}	48.61	206.55
His	448.30^{cC}	218.85^{dD}	464.26^{cB}	492.10^{eA}	117.83	492.10
Gly	105.79^{mB}	26.84^{lF}	69.73^{mC}	148.87^{lA}	14.67	148.87
Thr	60.36^{nD}	56.96^{jE}	132.59^{kB}	168.02^{kA}	15.12	168.02
Arg	307.24^{fC}	200.19^{eF}	332.67^{eB}	582.95^{dA}	126.24	582.95
Ala	260.48^{gB}	73.88^{iF}	225.23^{gC}	345.45^{gA}	24.54	345.45
Pro	2100.92^{aC}	1354.62^{aE}	3714.72^{aA}	3383.42^{aB}	650.29	3714.72
Tyr	185.16^{iH}	82.78^{hI}	201.35^{hG}	385.62^{fC}	28.02	530.47
Val	173.97^{jC}	47.94^{kFG}	190.90^{iB}	304.05^{hA}	18.74	304.05
Met	17.94^{pB}	25.38^{lA}	12.25^{oC}	6.27^{oF}	0.00	25.38
Cys	33.89^{oA}	ND	ND	ND	0.00	33.89
Ile	112.78^{lC}	53.79^{jE}	128.42^{kB}	186.07^{jA}	11.02	186.07
Leu	157.20^{kB}	55.63^{jE}	109.06^{lC}	212.60^{iA}	1.43	212.60
Trp	20.15^{pE}	14.29^{mF}	18.33^{nE}	26.50^{mD}	7.62	57.09
Phe	589.45^{bE}	349.36^{bG}	575.57^{bF}	1078.87^{bD}	86.64	2971.61
Orn	9.94^{qD}	10.23^{mD}	7.47^{oF}	16.27^{nA}	4.03	16.27
Lys	413.87^{dB}	286.71^{cCD}	424.79^{dB}	660.92^{cA}	242.31	660.92
Sum of EAA	**1764.79^{F}**	**972.84^{H}**	**1793.28^{E}**	**3028.92^{B}**	**439.24**	**4106.29**
Total	**5808.41^{D}**	**3207.65^{G}**	**7194.90^{B}**	**8958.19^{A}**	**1539.32**	**8958.19**

Means (n = 3), SD<10; within a column with small superscripts, within a row with capital superscripts differ significantly ($p < 0.05$).

Asp – aspartic acid, Glu – glutamic acid, Ser – serine, His – histidine, Gly – glycine, Thr – threonine, Arg – arginine, Ala – alanine, Pro – proline, Tyr – tyrosine, Val – valine, Met – methionine, Cys – cysteine, Ile – isoleucine, Leu – leucine, Trp – tryptophan, Phe – phenylalanine, Orn – ornithine, Lys – lysine; ND – not detected; EAA- Essential amino acids.

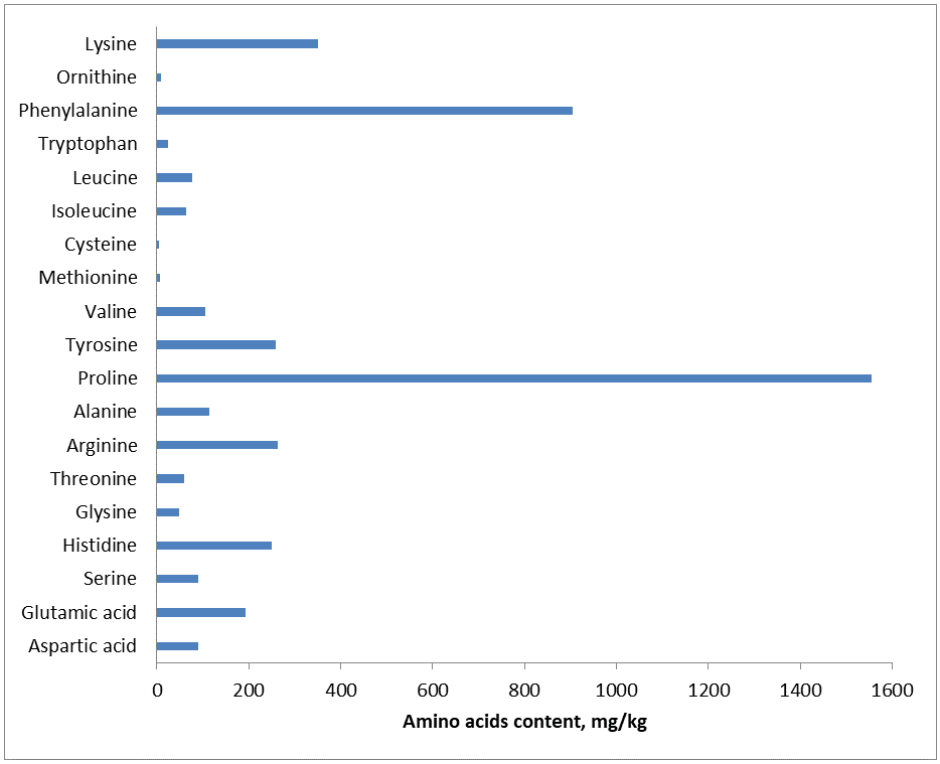

Figure 2: Total amino acids min, max and average values of 15 honey samples (mg/kg)

DISCUSSION

Quality parameters of mountain honey

Moisture content in quantitative terms is one of the most important components of honey. This parameter characterizes the quality of honey and can affect its storage. Honey samples with high moisture levels tend to ferment more easily. Considering that the moisture content of honey should generally be below 20% (Codex Alimentarius, 2001), the values obtained in this study were satisfactory, except of KS12.

The acidity of mountain honey is in accordance with the Codex standard and with findings of other researchers. The samples contained free acids in the range of 13.6–32.1 meq/kg. The average pH of the investigated honey was 4.4, with a typical range of 3.7–5.5. The Codex standard fixed a maximum acidity for honey of 50 meq/kg. According to Ciappini et al (2016) clover and eucalyptus honey from Argentina have an average of 19.5 and 22.3 meq/kg free acids, respectively. The pH of investigated samples was in good accordance with those obtained by Downey et al (2004). The study on honeydew and mixed honey in Spain has defined the free acidity as 15.87-35.66 meq/kg 22 (Table 1).

According to the Honey Quality and International Regulatory Standards, diastase activity must not be less than or equal to 8 diastase units (Codex Alimentarius 2001). The diastase number of the investigated honey samples was from 8.9 ± 0.1 to 77.9 ±5.1 in Schade units. The honey samples TO25 and AB24 had high diastase numbers (77.9±5.1 and 52.9±2.2 in Schade units, respectively), demonstrating the high quality of investigated mountain honey. For comparison, Bosnian and Herzegovinian honey had mean diastase activities of 10.2±8.3 (acacia) to 23.4±14.4 Schade units (blossom) (Ciric et al. 2018); honey from Argentina, 21.9±7.1 (clover honey) and 21.7±6.1 (eucalyptus honey) (Ciappini et al., 2016). The diastase number of honey from Greece was in the range of 8.1-15.0 DN (Pasias et al. 2017). In multifloral honey from Turkey, values in the range of 10.7-21.5 DN in Schade units were obtained with Phadebas methods (Gürbüz et al. 2020, Kivrak 2015).

The amino acids of honey samples from the mountain regions

The major AA of Kyrgyz honey are proline (1553 mg/kg), phenylalanine (905 mg/kg), lysine (349 mg/kg), arginine (261 mg/kg), tyrosine (258 mg/kg), and histidine (251 mg/kg), the last four AA are essential (Fig. 2). For comparison, in Turkish honeys, phenylalanine (4024 mg/kg), proline (1138 mg/kg), tyrosine (693 mg/kg), and isoleucine (749 mg/kg) (Kivrak 2015) were detected as the main AA. In acacia honey samples from China, proline, tyrosine, serine, alanine, and histidine were detected as the main AA (Sun et al. 2017), while proline, glutamic acid, phenylalanine, glycine, and serine accounted for the majority of AA in sunflower Serbian honey (Sakač et al. 2019). Other authors reported it as the main AA in Serbian unifloral honey proline, alanine, phenylalanine, threonine, and arginine (Kečkeš et al. 2013).

The proline content of the Kyrgyz honey samples accounted for 20%–50% of the total AA (Fig. 2). The proline content was the highest in samples received from pasture Kara-Shoro KS12 (3383 mg/kg) (Table 4). The samples AB24 (650 mg/kg) (Table 4) and TA24 (656 mg/kg) (Table 2) had a lower level of proline, but this level was in compliance with the defined minimum level for proline (Bogdanov et al. 1997). The proline content in honey harvested in 2005–2006 in Kyrgyzstan was 275–765 mg/kg (Smanalieva 2008). The proline content of Chilean honey was found in the range of 474–4421 mg/kg (Fuentes Molina et al. 2020). The proline content of honey from Serbia was in the range of 459 to 863 mg/kg (Sakač et al. 2019); from France, 208.7–592.3 mg/kg (Cotte et al. 2004) and from Spain 254 and 1992 mg/kg (Manzanares et al. 2014). Proline mainly comes from honey bee salivary secretions during the conversion of nectar or honeydew into honey and depends on the characteristic of slow or rapid honey harvest (Cotte et al. 2004).

The next most prominent AA in the investigated samples was the essential AA phenylalanine, which was found to be from 86 to 2971 mg/kg. The highest phenylalanine content of 2971 mg/kg was found in the sample AB23 (Table 4). In Turkish honey samples, phenylalanine was found as the main AA in the range of 499.6 to 15,047.6 mg/kg of honey. In addition, high levels of phenylalanine have been found in lavender, vitex, thyme, and sunflower honey (Kivrak 2015). Sun et al. (2017) reported that phenylalanine was the dominant AA in chaste honey samples, with a mean value of 1094.9 mg/kg (Sun et al. 2017). The phenylalanine content of lavender honey was found to be from 615 mg/kg (Hermosín et al. 2003) to 1152.5 mg/kg (Cotte et al. 2004).

A particular difference between Kyrgyz honey and Turkish honey is that lysine is the third major AA in Kyrgyz honey, where it was found in the range of 242 mg/kg (SU21) to 661 mg/kg (KS21) (Table 4), while in Turkish honey lysine was found in small amounts (Kivrak 2015).

The highest content of arginine was observed in KS12 (583 mg/kg) and the lowest in the sample AB 24 (216 mg/kg) (Table 4). The content of arginine in strawberry-tree (*Arbutus unedo* L.) honey was reported in the range of 8–39.8 mg/kg with an average value of 22.6 mg/kg (Afrin et al. 2017). Kivrak (2015) found arginine contents from 1.83 to 132.09 mg/kg in 17 Turkish monofloral honey.

Glutamic acid in honey samples was present in an amount from 65 mg/kg (AB24) to 590 mg/kg (KS12); it was higher than in (Cotte et al. 2004). Other acidic AA were found in a moderate amount. The sulphur-containing AA cysteine was found in only two honey samples AB23 (27.27 mg/kg), and SC22 (33.89 mg/kg). Methionine was the second-lowest amino acid in all of the Kyrgyz honey from 1.34 (SU21) to 25.38 mg/kg (SC25), except the sample KA21, in which methionine was not found. In honey collected in China and Estonia cysteine and methionine were not found (Rebane and Herodes 2008, Sun et al. 2017)

The total content of essential AA (histidine, threonine, arginine, tyrosine, valine, methionine, cysteine, isoleucine, leucine, tryptophan, phenylalanine, and lysine) was the highest in AB23 (4106 mg/kg) and is about two times of non-essentials, followed by TO11 (3143 mg/kg) and TO25 (3036 mg/kg). The content of valine, isoleucine, threonine and tryptophan ranged from 17 to 319 mg/kg, 11 to 186 mg/kg, 13 to 168 mg/kg and 0 to 58 mg/kg, respectively. Based on the calculated values, a higher percentage of essential AAs was found in TO11 (56%), CK21 (52%), AB23 (66%) and KA21 (59%), indicating a rich source of essential AAs that are fundamental for daily diet and human growth.

The highest content of total AA was in the KS12 (8958 mg/kg) sample, and the lowest content was in AB24 (1539 mg/kg), with a mean value for all examined samples of 4470 mg/kg (Fig. 2). The

results of amino acid contents were comparable with those obtained by Sun *et al.* (2017) for honey from China, the total amount of amino acids ranged from 3974 mg/kg (rape honey) to 7191 mg/kg (jujube honey). Other authors reported maximum values of AA as 2874.1 mg/kg (Hermosín et al. 2003) and 1831.6 mg/kg for lavender honey (Cotte et al. 2004). The mean content of free amino acids in honeydew honey was 398.41 mg/kg, in the group of buckwheat 633.5 mg/kg, golden-rod 425.93 mg/kg, and heather honey 414.49 mg/kg (Janiszewska et al. 2012).

As the main source of amino acids in honey is pollen (Cotte et al. 2004), it can be assumed that Kyrgyz honey samples are collected from flowers rich in pollen. The high proline content of the examined samples could be related to the arid climatic conditions of Kyrgyzstan. The air temperature in Kyrgyzstan in summer is +27°C on average. The average minimum temperature is +16°C and the average maximum temperature is kept at +33°C. Plant nectar may have low moisture content in the hot and dry climatic conditions of the mountains, and the bees may have to process the nectar more with saliva to suck it from the plant. Additionally, according to beekeepers of Kyrgyzstan, in the mountains usually the honeybees have a longer distance from forage to the hive, than in lowlands with agricultural plants. As a result, the collected nectar content of proline in mountain honey increases.

Effect of altitude on the amino acid composition of honey

A comparative analysis of individual AA and total AA between samples was performed using Duncan's test. The content of total AA in samples collected from the same region but from different seasons are not the same (TO25 and TO11). Also, differences in total AA content were observed in honey obtained from the same region and in the same collection seasons (KS11 and KS12, AB23 and AB24, SC25 and SC22). Weak positive correlations were found between the altitude of the collection area and asparagine, glutamine, histamine, glycine, threonine, alanine, proline, valine, and total amino acidity. The Pearson correlation coefficient is shown in Table 5. Thus, differentiation between mountain honey from different altitudes on the basis of the amino acid composition appears to be feasible. According to many researchers, the proteins and AA present in honey are of both animal and plant origin, which leads to variability of amino acid contents in honey from the same flower (Chua et al. 2015, Sun et al. 2017). Therefore the high variability of AA content of the samples could be related to the rich biodiversity of the mountain landscape and the long distance to the hive.

Table 5. Pearson's correlation coefficient of altitude of the collection area and individual amino acids

Amino acid	Pearson Correlation
Asp	0.288**
Glu	0.347**
Ser	0.456**
His	0.406**
Gly	0.366**
Thr	0.485**
Arg	0.302**
Ala	0.396**
Pro	0.477**
Tyr	0.468**
Val	0.364**
Met	0.294**
Cys	NS
Ile	0.465**
Leu	0.334**
Trp	NS
Phe	0.329**
Orn	0.350**
Lys	NS
Sum of EAA	0.440**
Total	0.515**

** Correlation is significant at the 0.01 level (2-tailed). Asp – aspartic acid, Glu – glutamic acid, Ser – serine, His – histidine, Gly – glycine, Thr – threonine, Arg – arginine, Ala – alanine, Pro – proline, Tyr – tyrosine, Val – valine, Met – methionine, Cys – cysteine, Ile – isoleucine, Leu – leucine, Trp – tryptophan, Phe – phenylalanine, Orn – ornithine, Lys – lysine; NS – not significant; EAA- Essential amino acids.

Correlation of amino acid content with other chemical parameters

In this work, the correlation between pH, diastase activity, moisture content, acid content and amino acid content was investigated (Table 6). Unfortunately, no statistically significant correlation was observed between AA and pH ($r = 0.284$, $p = 0.585$) and AA and diastase number ($r = -0.074$, $p = 0.890$), AA with acidity ($r = -0.429$, $p = 0.05$). However, the amino acid composition of honey was positively correlated with the moisture content of honey ($r = 0.905$, $p = 0.05$). The linear relationship between total AA and moisture content could be explained by the solubility of AA in water.

Table 6. Pearson's Correlation coefficients of total amino acid content with other chemical parameters

	Moisture content, g/100 g	Acidity, g/100 g	pH	Diastase number, Shade Unit	Amino acid, mg/kg
Moisture content, g/100g	1				
Acidity, g/100g	-.563	1			
pH	0.432	0.177	1		
Diastase number, Shade Unit	-0.324	0.465	0.011	1	
Amino acid, mg/kg	0.905*	-0.429	0.284	-0.074	1

* Correlation is significant at the 0.05 level (2-tailed).

Conclusion

The study showed that the quality parameters of investigated honey samples such as water content, organic acids, and pH met international standards. A total of 19 AA using HPLC in Kyrgyz honey were determined. The content of individual AA and also total AA in samples collected from the same region and the same collection seasons were different. The major AA of mountain honey were proline, phenylalanine, lysine, histidine, tryptophan, and tyrosine. The high variability and high AA content of the samples could be related to the biodiversity of the mountain landscape. The correlation among diastase activity, moisture content, acid content and AA content revealed that the AA composition of honey is positively correlated with the moisture content of honey. Obtained results provides useful information for the characterization of honey from the mountainous regions of Kyrgyzstan. Further studies of antioxidant activities of mountain honey and their correlation with amino acids and also total polyphenol content are needed to contribute to the limited literature on mountain honey.

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Code availability: Not applicable

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