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Free Amino Acids Content of Honeys from Poland

Katarzyna Janiszewska[1]*, Magda Aniołowska[2], Piotr Nowakowski[1]

*[1]Institute of Animal Breeding, Wrocław University of Environmental and Life
Sciences, ul. J. Chełmońskiego 38 C, 51–630 Wrocław, Poland
[2]Faculty of Chemistry, Wrocław University of Technology, C.K. Norwida 4/6, 50–373 Wrocław, Poland*

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The objective of this study was to investigate free amino acids composition of Polish honeys with different botanical origin. Honeys (n=18) with dominant buckwheat, raspberry, acacia, heather and goldenrod pollen, and honeydew honey were analysed. For determination of free amino acids liquid chromatography methods were applied. Identification of 25 free amino acids was performed. Considerable variation in the total content of free amino acids ranging from 186.19 mg/kg to 921.08 mg/kg was stated. The dominant free amino acid in all types of honey was proline with the highest detected amount in one sample of heather honey 387.88 mg/kg. As an indicator of honeys with predominant raspberry and buckwheat pollen high concentrations of aspartic acid and asparagine (accounting for *ca*. 20 and more mg/kg) are suggested. The content of tyrosine, leucine, isoleucine and valine ranging from 10 to *ca*. 20 mg/kg was characteristic of raspberry and at concentrations above 20 mg/kg of buckwheat honeys. The cluster analysis showed the closest correlation between heather and goldenrod honeys. The largest distance was stated between buckwheat and all other honey groups. The results show that it was impossible to clearly distinguish the botanical origins of Polish honey samples based on their amino acid composition.

INTRODUCTION

There are many potential analytical markers for the distinction of the botanical origin of honey. Melissopalinology has been in use for years but nowadays it is assumed that such a procedure has severe drawbacks [Nozal *et al.*, 2004]. The most frequently aroma compounds, sugar profile, flavonoids pattern, organic acids, isotopic relations, protein and amino acid composition are analysed to characterise honey [Anklam, 1998; Bogdanov *et al.*, 2004; Nozal *et al.*, 2004; Ruoff & Bogdanov, 2004; Wilczyńska, 2010]. Amino acids markers were described by Gonzales Paramas *et al.* [2006] who showed that the next abundant after proline are: γ-aminobutyric acid (g-Aba) for chestnut-tree and oak honeys and asparagine for ilex. Distinction of origin is not an easy task because honey composition is affected by many factors, including climatic and soil conditions as well as storage, aging and processing techniques [Hermosin *et al.*, 2003]. European Union regulations concerning food products [Council Directive, 2001; Codex Alimentarius, 2001] have established composition and quality parameters for honey but those do not involve the content of free amino acids. There is no yet approved method that would state how to determine amino acid content of honey [Rebane & Herodes, 2010].

Amino acids play a central role as building blocks of proteins and as intermediates in the metabolism. They are precursors for the production of the key flavour compounds. Proteins and amino acids occurring in honey are of both animal and plant origin. Since pollen is the main source of amino acids, its profile could be useful to characterise the botanical origin of honey, however free amino acids are added by bees themselves and this leads to a high variability of the amino acid content within honeys from the same botanical source [Bogdanov & Martin, 2002].

Most of amino acids in honey are in the bound form and free amino acid content may be as low as one fifth of the total [Gonzales Paramas *et al.*, 2006]. Free amino acids are a minor but important component of honey. The occurrence of 27 free amino acids in honey was affirmed by Hermosin *et al.* [2003].

The major amino acid in honey is proline (50–85%). However variations of proline content in different unifloral honeys are quite large and it is not possible to classify unifloral honey on the basis of this parameter only. This amino acid comes mainly from the honeybee during the conversion of nectar into honey. Together with other factors related to bees, such as sacharase and glucose oxidase activities, may constitute an indicator of honey ripeness [Bogdanov *et al.*, 2004]. Proline in honey ought to account for more than 200 mg/kg [Hermosin *et al.*, 2003].

Methods of honey amino acids analysis are still discussed. Gas chromatographic and liquid chromatographic methods have been used most frequently for profiling amino acids

* Corresponding author:
  E-mail: janiszewska.katarzyna@gmail.com (K. Janiszewska)

analysis [Davies, 1975; Louveax *et al.*, 1978; Conte *et al.*, 1998; Nozal *et al.*, 2004; Iglesias *et al.*, 2004].

The aim of the present research was to evaluate the content of free amino acids in different types of Polish honeys and to relate their compositions to the botanical origin of honey.

MATERIALS AND METHODS

Honey samples

The material consisted of 18 samples of honey which were purchased directly from beekeepers from Poland (mainly Lower Silesia) during 2 consecutive seasons. Honeys derived from different main crops with domination of buckwheat (n=4), raspberry (n=3), acacia (n=3), heather (n=3), goldenrod (n=2) and honeydew (n=3). To confirm the main source of nectar in honeys melissopalynological analyses were performed. The pollen analysis was carried out according to the International Commission of Bee Botany of International Union of Biological Sciences described by Louveaux *et al.* [1978]. Honeydew and floral honeys were differentiated using their electric properties (methodology proposed by Łuczycka [2009]).

Sample preparation

Sample preparation was conducted in accordance with the methodology proposed by Gonzalez Paramas *et al.* [2006]. For the separation of sugars a glass column (diameter 14 mm) filled with DOWEX 50Wx8–200 resin (Fluka) was used. Weighted amount (1 g) of honey was dissolved in 10 mL of 0.01 mol/L phosphate buffer (pH 2.12) and transferred to the top of the column. At first sugars were eluted with 3 x 10 mL of water, then amino acids were eluted with 15 mL 7N NH_4OH

TABLE 1. Mobile phase flow rate.

Time (min)	Column T (ᵒC)	Buffer No.
0.00	40	1
2.00	45	2
5.00	45	2
25.00	50	2
45.00	60	2
55.00	70	3
60.00	70	3
81.00	70	4
115.00	60	5
155.00	60	5
169.00	60	5
177.00	60	6
180.00	60	1
185.00	55	1
201.00	45	1
205.00	40	1
209.00	40	1

Buffers: 1: pH 2.70; 2: pH 2.90; 3: pH 3.6; 4: pH 3.0; 5: pH 4.65; 6: 0.3M LiOH.

and 10 mL of water. The sample was evaporated to dry matter at a temperature below 50°C under the vacuum. Dry matter was redissolved in a measuring flask with 10 mL of 0.1 N lithium buffer (pH 2.2) and measurement was achieved.

Analytical method

The content of amino acids was determined using automatic amino acid analyser (AAA-400, INGOS, Czech Republic) equipped with Ostion LG FA ion-exchange column (200 x 3.7 mm, INGOS). Mobile phase was lithium buffers system with pH 2.7–4.64. The flow rate of particular buffers was based on instructions described by INGOS and attached to amino acid analyser program (Table 1). The detection was monitored at two wavelengths, *i.e.*: 440 and 570 nm after a reaction of eluate with ninhydrin (buffered at pH 5.5). The rate flow of lithium buffers was 0.2 mL/min and that of ninhydrin solution – 0.3 mL/min. Column temperature was kept at 40–70°C and detector at 121°C. Time of one analysis was 209 min.

Calculations were performed with computer program CHROMULAN (Pikron, Czech Republic).

Statistical analysis

Differences in free amino acids content between honeys were confirmed using one way analysis of variance and Duncan's test at a significance level p≤0.05. Cluster analysis was used for the general characteristics of honeys. Tree diagram (cluster) – complete linkage as a connection method and Euclidean distance was drawn based on the average percentage content of individual amino acids in honeys [Statistica v. 9].

RESULTS AND DISCUSSION

Melissopalynological microscopic analysis confirmed the main plant source of the investigated honeys. The content of pollen of the dominant plant in the analysed samples was presented in Table 2. Buckwheat honey contained 30–51%

TABLE 2. Percentage of predominant pollen in the analysed floral honeys.

Sample number	Origin of predominant pollen	Type of honey	Predominant pollen (%)
1			30
2			42
3	*Fagopyrum esculentum* M.	buckwheat	47
4			51
5			18
6	*Rubus ideaus* L.	raspberry	21
7			22
8			17
9	*Robinia pseudoacacia* L.	acacia	21
10			30
11			52
12	*Calluna vulgaris* L.	heather	58
13			60
14			45
15	*Solidago canadensis* L.	goldenrod	53

of *Fagopyrum esculentum* Moench grains, raspberry honey – 18–22% of *Rubus ideaus* L. grains, acacia honey – 17–30% of *Robinia pseudoacacia* L. grains, heather honey – 52–60% of *Calluna vulgaris* L. grains, and goldenrod honey 45–53% of *Solidago canadensis* L. grains. The analysis of electric properties (permittivity, dielectric loss coefficient and conductivity) confirmed differences between honeydew and floral honeys (data not shown).

The applied method allowed the separation and quantification of 25 free amino acids (Table 3). Considerable variation in the total content of free amino acids ranging from 186.19 mg/kg (raspberry honey) to 921.08 mg/kg (buckwheat honey) was stated between single samples. The average content of the total free amino acids in the tested groups of honeys ranged from 390.63 mg/kg (acacia) to 633.50 mg/kg (buckwheat). Despite large differences in the mean total content of free amino acids statistically significant differences were not confirmed – standard variation obtained confirmed a lack of uniformity within the group of honeys.

Generally, floral honeys are characterised with a lower content of amino acids than honeydew honeys [Iglesias *et al.*, 2004; Kędzia & Hołderna-Kędzia, 2008]. However this tendency was not observed in the samples analysed in our study. Our results showed that the mean content of free amino acids in honeydew honey (398.41 mg/kg) was lower than in the group of buckwheat (633.5 mg/kg), goldenrod (425.93 mg/kg) and heather honeys (414.49 mg/kg). It may, thus, be assumed that the amount of free amino acids in floral honeys is lower than 1000 mg/kg. Fine examples to this point could be Polish buckwheat (720 mg/kg), German multifloral (277 mg/kg) and heather honeys (368 mg/kg) [Kędzia & Hołderna-Kędzia, 2008]. The total free amino acids content of honeydew honeys in some cases exceeds 1000 mg/kg (Italian honey from spruce honeydew – 2690 mg/kg, Spanish honeys from rose and raspberry honeydew – mean 2562 mg/kg) while in others is much lower (350 mg/kg in German honeydew honey and 540 mg/kg in Italian pine honeydew honey) [Kędzia & Hołderna-Kędzia, 2008]. Those lower values are in accordance with our own results (mean 398.41 mg/kg).

The predominant amino acid in all tested groups of honey was proline (the highest detected content of 387.88 mg/kg in one of heather honeys tested). Kędzia & Hołderna-Kędzia [2008] suggest that proline constitutes about 57% and together with phenylalanine almost 66% of the total free amino acids. However phenylalanine content in our studies did not exceed 6.65% and on average was at the level of *ca*. 2.0%. Proline content in sources cited by Kędzia & Hołderna-Kędzia [2008] ranged from 311 to 1232 mg/kg, including Polish buckwheat honey with 317.80 mg/kg, whereas in our study in this group of honey the mean value (4 samples) reached only 227.83 mg/kg. Two heather honeys studied showed proline content higher than 300 mg/kg while another one of only 130 mg/kg. Other authors stated 280 mg/kg of proline in strawberry-tree honey [Spano *et al.*, 2009] and 467 mg/kg in heather honey [Nozal *et al.*, 2004]. Comparisons made above showed that proline content could not be a good indicator of the botanical origin of honey. A specific relation between the amount of this

amino acid and plant source was not observed in our studies. At the same time, this confirms that proline is mainly added by bees in the process of nectar transformation to honey. Proline content lower than 180 mg/kg [Bogdanov & Martin, 2002] or 200 mg/kg [Hermosin *et al.*, 2003] could mean that the honey had been adulterated with sugar. Proline content could be one of parameters in determination of honey authenticity.

Amino acids found in high levels in almost all groups of honeys were: asparagine, valine, isoleucine, leucine, tyrosine, phenylalanine – average content in different honeys from 4 to over 85 mg/kg. Lower but also important amounts of threonine, serine, glutamic acid, glutamine, alanine, β-aminobutanoic acid, lysine (ranging from *ca*. 3 to 10 mg/kg) were detected in the study (Table 3).

Raspberry honeys showed, beside proline, a quite high amount of asparagine (28.53 mg/kg) and aspartic acid (22.41 mg/kg) – Table 3. Raspberry honeys contained also leucine, tyrosine and isoleucine in a significant concentration (up to 50 mg/kg in a single sample). Apart from proline, the buckwheat honeys showed clearly the predomination of leucine (85.54 mg/kg), isoleucine (55.76 mg/kg) and tyrosine (51.21 mg/kg). Like in raspberry honeys, here also contents of asparagine and aspartic acid were high. In acacia honeys (except for proline) we specified tyrosine (51.01 mg/kg), glutamine (18.58 mg/kg) and glutamic acid (9.12 mg/kg) as predominant amino acids. In the heather honeys, besides a high content of proline (284.09 mg/kg) and phenylalanine (16.01 mg/kg), contents of other single amino acids did not exceed 10 mg/kg. Goldenrod honeys were characterised with a high content of phenylalanine (13.69 mg/kg). Other significant amino acids in this group of honey were glutamine, lysine, β-alanine, alanine, leucine and tyrosine in descending order from 10.43 to 8.17 mg/kg. In honeydew honeys we observed (beside proline) a high content of aspartic acid, serine, glutamine, glutamic acid, alanine, valine, leucine and tyrosine at the level ranging from 9 to 12 mg/kg (Table 3).

Arginine and tryptophan were not crucial for the discrimination between honeys in our study but they have been shown to be characteristic for some types of floral honey [Spano *et al.*, 2009]. In strawberry-tree honey analysed by Spano *et al.* [2009] the proportions of the most important amino acids (predominant content of proline and significant presence of glutamic acid, arginine, alanine and phenylalanine) were similar to our results for floral honeys, especially for goldenrod and heather honeys, with exception of arginine which was not as important as other amino acids.

The statistical analysis showed significant differences (p≤0.05) in concentrations of individual free amino acids between the honeys only in the case of asparagine, valine, isoleucine, leucine, β-aminobutanoic acid, lysine and histidine. Asparagine content was significantly higher in raspberry and buckwheat honey. In buckwheat honey the concentrations of such amino acids as valine, isoleucine and leucine were significantly higher than in the other honey samples. Honeydew honey showed a significantly lower content of lysine, whereas honey from heather and goldenrod contained a significantly higher content of histidine than the other honey types (Table 3).

TABLE 3. Content of free amino acids in Polish honeys (mg/kg).

	Acacia (n=3)	Raspberry (n=3)	Buckwheat (n=4)	Honeydew (n=3)	Heather (n=3)	Goldenrod (n=2)
	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD
Asp	7.77±2.21	22.41±24.35	30.07±18.63	12.16±10.13	6.39±3.02	4.28±0.29
Thr	3.33±1.50	4.52±3.71	10.00±5.30	4.04±2.82	5.51±3.99	5.58±3.15
Ser	6.20±1.38	8.82±8.40	15.77±7.02	9.58±7.35	6.72±3.52	7.87±3.71
Asn	7.16[b]±3.10	28.53[a]±13.90	22.14[ab]±16.52	3.23[b]±1.47	5.80[b]±3.70	3.33[b]±0.21
Glu	9.12±3.17	7.56±5.27	13.62±10.22	11.40±9.30	9.10±2.94	5.65±0.84
Gln	18.58±21.07	8.99±4.85	12.43±11.06	12.53±7.28	7.56±4.05	10.43±4.93
α-Aaa	1.11±0.13	1.09±0.32	2.97±3.75	1.03±0.58	1.33±0.26	1.07±0.17
Pro	225.74±43.88	189.13±103.98	227.84±78.30	263.36±96.46	284.09±135.64	292.93±57.88
Gly	2.79±0.73	4.14±2.79	8.46±5.48	2.27±2.08	4.08±2.00	3.32±1.87
Ala	7.26±1.36	7.90±4.49	13.05±7.61	9.16±3.48	9.75±4.19	9.02±2.95
α-Aba	2.22±0.73	7.67±9.31	7.20±7.63	6.49±4.26	1.09±0.77	0.66±0.72
Val	5.67[b]±1.97	12.70[b]±13.39	34.63[a]±20.56	9.42[b]±3.89	6.83[b]±3.35	7.14[b]±3.74
Met	1.58±0.32	4.00±3.65	6.19±3.92	3.32±2.85	1.03±0.60	1.17±0.09
Ile	2.83[b]±1.21	14.52[b]±16.50	55.76[a]±35.43	7.46[b]±2.63	4.09[b]±2.71	6.67[b]±5.72
Leu	2.41[b]±0.73	21.80[b]±24.48	85.54[a]±58.13	9.26[b]±6.79	5.26[b]±3.83	8.39[b]±7.62
Tyr	51.01±78.85	16.47±14.95	51.21±32.08	9.39±8.62	4.97±1.80	8.17±5.15
Phe	6.44±1.58	12.05±8.10	10.39±2.24	5.77±2.93	16.01±16.03	13.69±5.71
β-Ala	6.24±1.92	4.65±3.10	9.95±11.68	5.05±2.73	8.31±4.59	9.45±2.21
β-Aba	4.64[ab]±1.51	4.52[ab]±1.06	3.17[ab]±2.11	2.57[b]±0.41	5.71[a]±0.93	4.24[ab]±0.80
γ-Aba	3.90±0.98	3.86±0.83	3.61±0.78	2.55±0.43	3.72±1.43	3.95±0.70
EA	4.09±1.96	2.40±0.35	3.32±2.58	2.08±0.33	2.35±0.53	2.78±0.29
Orn	0.67±0.45	0.22±0.10	0.49±0.43	0.64±0.32	0.50±0.35	0.52±0.05
Lys	6.51[ab]±4.58	3.43[ab]±1.66	4.06[ab]±1.43	2.91[b]±0.81	8.19[ab]±6.10	10.22[a]±3.42
His	1.45[b]±1.04	0.89[b]±0.31	1.24[b]±0.68	0.86[b]±0.03	2.57[ab]±1.87	3.97[a]±1.97
Arg	1.90±1.05	0.64±0.43	0.41±0.09	1.90±2.09	3.55±3.06	1.46±0.73
Total	390.63±105.34	392.90±206.47	633.50±270.08	398.41±135.52	414.49±193.82	425.93±113.37

[a,b] – significant differences between the mean contents of individual amino acids compared in a row, means denoted by different letters differ at p≤0.05.

Amino acids abbreviations: Asp – aspartic acid, Thr – threonine, Ser – serine, Asn – asparagine, Glu – glutamic acid, Gln – glutamine, α-Aaa – alpha-amino adipic acid, Pro – proline, Gly – glycine, Ala – alanine, α-Aba – alpha-amino butyric acid, Val – valine, Met – methionine, Ile – isoleucine, Leu – leucine, Tyr –tyrosine, Phe – phenylalanine, β-Ala – beta-alanine, β-Aba – beta-amino butyric acid, γ-Aba – gamma-amino butyric acid, EA – ethylamine, Orn – ornithine, Lys –lysine, His – histidine, Arg – arginine.
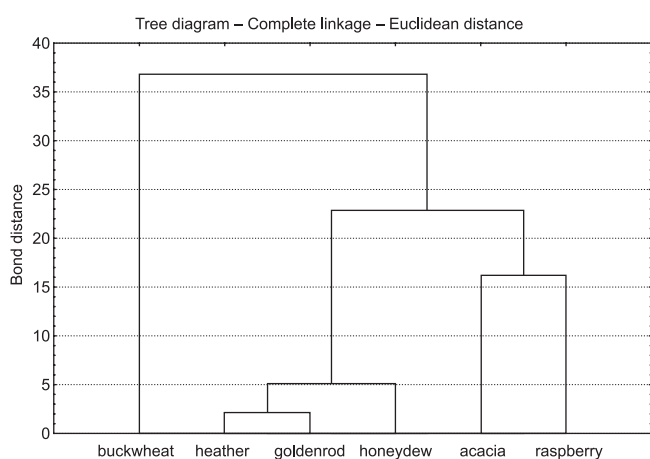

FIGURE 1. Tree diagram of the average percentage of individual free amino acids in honeys.

The cluster analysis performed (tree diagram – Figure 1) was based on the average percentage of individual amino acids in honeys. The closest correlation was stated between heather and goldenrod honeys (bond distance – 2.21). It may be suggested that similar phenological terms of these plants flowering (August/September) may have an impact on similar amino acid composition of the nectar, and thereby on a similar composition of free amino acids in honeys with predomination of these plants. Some similarity in amino acids composition also exists between honeys from acacia and raspberry which bloom at the same time (May/June). Relatively close to heather and goldenrod honeys was the composition of honeydew honey (bond distance – 5.20). The largest distance (36.76) in the cluster analysis was stated between buckwheat and all other honey types. It means that free amino acid composition of this honey differs significantly from the others.

SUMMARY AND CONCLUSIONS

The average content of the total free amino acids in tested honeys ranged from 390.63 mg/kg (acacia) to 633.50 mg/kg (buckwheat). Amino acids found in high levels (except proline) in almost all types of honeys were asparagine, valine, isoleucine, leucine, tyrosine, phenylalanine – the average content in different honeys ranged from 4 to over 85 mg/kg. Phenylalanine is the second most abundant amino acid generally in honeys but it was not the case in our study except heather and goldenrod honeys. In turn, high concentrations of aspartic acid and asparagine (ranging *ca.* 20 and more mg/kg) are suggested as an indicator of raspberry and buckwheat honeys. The contents of tyrosine, leucine, isoleucine and valine ranging from 10 to *ca.* 20 mg/kg was characteristic for raspberry and at concentrations above 20 mg/kg for buckwheat honeys. The total content of free amino acids as well as free amino acid composition of buckwheat honey differed and distinguished this source of honey from the others. The results show that it was impossible to clearly distinguish the botanical origins of Polish honey samples based on their amino acids composition due to their high variability.

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