Free amino acids profile of Polish and Slovak honeys based on LC–MS/MS method without the prior derivatisation

Stanisław Kowalski² · Mária Kopuncová^{1,3} · Zuzana Ciesarová¹ ·
Kristína Kukurová¹

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Abstract LC–MS/MS method was applied for determination of free amino acids in honey without derivatisation steps. Twenty free amino acids including aspartic acid, asparagine, glutamic acid, glutamine, alanine, arginine, glycine, leucine, histidine, hydroxyproline, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine and ornithine were analyzed in thirty honey samples from Poland and Slovakia. The analysis covered: acacia, lime, rape, multifloral and forest types of honey. Applied method was characterized to had good sensitivity with limit of detection ranged from 3.0 ng/cm³ for valine to 13.0 ng/cm³ for hydroxyproline. Average content of proline (main amino acid component in honey) ranged from 151.46 µg/g (rape honey from Slovakia) to 389.66 µg/g for forest honey (honeydew honey) from Poland. In analyzed honeys large quantities of glutamine, glutamic acid, lysine, phenylalanine, asparagine, alanine, and valine were also found.

Keywords Free amino acids · Honey · Chromatographic analysis

Introduction

Honey is sweet natural substance which main components are monosaccharides—glucose and fructose. Besides carbohydrates, other compounds such as proteins, organic acids, minerals and others come into its composition (Da Silva et al. 2016). Protein content is highly related to bee species. It was found that honeys produced by *Apis mellifera* are poorer in proteins compared to those obtained by the bee species *Apis cerana* (Won et al. 2009). Proteins and amino acids come both from plant and animal sources. They are present in the nectar or honeydew and also in secretions of bees. However, it is believed that the main source of protein and amino acids in honey is bee pollen (Da Silva et al. 2016). Amino acids represent about 1% (w/w) of honey ingredients. Qualitative profile of amino acids in honey include: proline, glutamic acid, aspartic acid, glutamine, histidine, glycine, threonine, β-alanine, arginine, α-alanine, valine, methionine, cysteine, isoleucine, leucine, tryptophan, phenylalanine, ornithine, lysine, serine, asparagine and others (Hermosin et al. 2003; Kečkeš et al. 2013). The proline takes the largest quantity up to 80% of all amino acids (Hermosin et al. 2003) and its content should be at least 180 µg/g. Lower values indicate immaturity or falsification of honey with sugar (Bogdanov et al. 1999).

Because pollen is considered to be the main source of amino acids in honey, analysis of its content together with chemometric techniques can be used for estimation of botanical and geographical origin of honeys. There are many reports regarding the use of this analysis for the characterization of honey from different countries (Kečkeš et al. 2013; Hermosin et al. 2003; Zieliński et al. 2014; Chen et al. 2017). On the other hand, bees also contribute to free amino acids content in honey which results in high

✉ Stanisław Kowalski
rrkowals@cyf-kr.edu.pl

¹ National Agricultural and Food Centre, Food Research Institute, Priemyselná 4, 82475 Bratislava, Slovak Republic

² Department of Carbohydrates Technology, Faculty of Food Technology, University of Agriculture in Krakow, ul. Balicka 122, 30-149 Kraków, Poland

³ Institute of Chemistry, Faculty of Natural Science, Comenius University, Ilkovičova 6, 84215 Bratislava, Slovak Republic

variability of particular components in honeys of the same botanical origin (Bogdanov and Martin 2002). Amino acid content in honey may be also subject to some natural fluctuations due to the Maillard reactions taking place between them and monosaccharides as well as due to the action of particular enzymes as proteases and peptidases. During approximately 9 months of storage a level of the amino acid content can undergoes some changes but after that amino acid profile is more stable (Iglesias et al. 2006). Taking this above consideration it seems to be clear that this parameter itself does not provide conclusive information about botanical origin of honey (Bogdanov and Martin 2002).

The analysis of amino acids in honey is usually connected with derivatisation steps (Nozal et al. 2004; Pereira et al. 2008; Kelly et al. 2010; Rebane and Herodes 2010) which makes it more complicated and more reagent-consuming. However, an application of a mass spectrometry (MS) for detection of amino acids allows also their direct analysis in food extracts without the use of derivatisation step. The liquid chromatography–mass spectrometry (LC–MS) with atmospheric pressure chemical ionization (APCI) in positive mode was used by Özcan and Şenyuva (2006) for the determination of amino acid in several foodstuffs including different fruit juices, baby food, milk, beer, wine and nuts. Another methods applying the liquid chromatography–tandem mass spectrometry (LC–MS/MS) with positive electrospray ionization (ESI) were described for analysis of amino acids in plant extracts (Thiele et al. 2012), orange juices (Gómez-Ariza et al. 2005) and bakery products (Ciesarová et al. 2009).

The aim of this study was to characterize the profile of free amino acids of Polish and Slovak honeys using a direct method of amino acids determination in honey via LC–MS/MS. An attempt was made for diversification of honeys according to botanical as well as geographical origin.

Materials and methods

Materials

Standards of amino acids: L-aminoacids kit containing p.a. standards (98%) of Aspartic acid (Asp), Asparagine (Asn), Glutamic acid (Glu), Glutamine (Gln), Alanine (Ala), Arginine (Arg), Glycine (Gly), Leucine (Leu), Histidine (His), Hydroxyproline (Hyp), Isoleucine (Ile), Lysine (Lys), Methionine (Met), Ornithine (Orn), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Trp), Valine (Val), were purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany), internal standard was *d*₃-Glu (*d*₃-glutamic acid), (97%), (Cambridge Isotope Laboratories, Andover, USA).

Thirty samples of honeys with guaranteed botanical origin were obtained from individual small producers in Poland (three manufacturers fifteen samples of honey) and Slovakia (three manufacturers fifteen samples of honey). The set covered acacia, lime, rape, multifloral and forest honeys.

Methods

Basic characteristic of honeys quality

To determine the quality of analyzed honey, the hydroxymethylfurfural (HMF) content, diastatic activity (diastase number—DN) as well as water content were determined. Analysis were carried out according to methods of International Honey Committee (IHC) (Bogdanov et al. 1997).

LC–MS/MS analysis of free amino acids

Sample preparation 1 g of honey was transferred into a 50 mL tube with a cap. A volume of 9.95 mL of 0.1% (v/v) acetic acid and 50 µL of the internal standard working solution (*d*₃-glutamic acid) was added to the samples and the mixture was vortexing for 2 min. The solution was filtered through 0.45 µm pore size nylon syringe filter prior to LC–MS analysis.

Chromatographic analysis The Agilent 1200 series (Agilent, Technologies, Waldbronn, Germany) chromatograph coupled with Agilent 6410 Triple Quad LC/MS detector (Agilent Technologies, Palo Alto, California, USA) was used for separation of free amino acids. Mobile phase was a mixture of 100 mL acetonitrile (Sigma-Aldrich, Steinheim, Germany), 1 mL of acetic acid (Fischer Scientific, Loughborough, UK) and 500 mL of 0.05 mM water solution of perfluorooctanoic acid (96%), (Sigma-Aldrich, Steinheim, Germany). Separation was achieved with Purospher Star RP-8ec column (150 mm × 4.6 mm × 3 µm, Merck, Darmstadt, Germany) at temperature of 25 °C, and flow rate of 0.5 mL/min.

Detection was done in MRM mode based on in-source generation of the protonated molecular ions of the amino acids and the internal standard (*d*₃-glutamic acid), as well as collision-induced production of amino acid-specific fragment ions. The following instrumental parameters were used for LC–MS/MS analysis of amino acids in the positive MRM mode: nitrogen as a drying gas (320 °C) with flow rate 8.0 L/min, nebulizer pressure 50 psi and capillary voltage 3 kV, the specific MRM amino acid transitions as well as their corresponding fragmentor voltages, collision energies and dwell are summarized in Table 1.

Preparation of standard solutions of amino acids and internal standard Mixed stock solution of amino acids

Table 1 MRM amino acid transitions and corresponding fragmentor voltages. Collision energies and dwell

Optimized MRM parameters									Parameters of calibration		
Amino acid	Time segment	Precursor ion	Production		Fragmentor [eV]	Collision energy [eV]		Dwell [ms]	R ²	LOD [ng/ml]	LOQ [ng/ml]
			Quantifier	Qualifier		Quantifier	Qualifier				
Ala	1	90	44	90	50	6	2	20	0.9990	10	30
Arg	3	175	70	175	100	25	5	50	0.9992	10	30
Asn	1	133	74	87	50	12	5	20	0.9998	8	25
Asp	1	134	74	88	50	12	5	20	0.9996	10	30
Gln	1	147	84	130	50	15	5	20	0.9919	10	30
Glu	1	148	84	102	50	15	10	20	0.9994	6	20
Gly	1	76	76	30	50	2	6	20	0.9997	11	33
His	3	156	110	156	100	15	2	50	0.9996	10	29
Hyp	1	132	86	132	50	15	2	20	0.9998	13	39
Ile	2	132	86	132	80	5	2	50	0.9997	8	24
Leu	2	132	86	132	80	5	2	50	0.9997	11	33
Lys	3	147	84	130	80	15	5	50	0.9991	7	23
Met	2	150	56	133	80	15	5	50	0.9972	4	12
Orn	3	133	116	70	80	5	20	50	0.9997	9	28
Phe	2	166	120	103	80	10	30	50	0.9997	10	30
Pro	1	116	70	116	50	10	0	5	0.9978	9	29
Ser	1	106	60	106	50	5	2	20	0.9987	10	30
Thr	1	120	56	74	50	15	8	20	0.9978	8	26
Trp	3	205	146	188	100	15	5	50	0.9998	11	34
Val	2	118	72	118	80	10	2	50	0.9994	3	10

and a stock solution of an internal standard were prepared in concentration 1000 µg/mL of acidified water (0.1% acetic acid).

Working solutions of amino acids and the internal standard prepared by 10 time dilution of stock solutions in acidified water was 100 µg/mL.

A calibration curve of amino acids was prepared in the range from 0.02 to 6 µg/mL, internal standard concentration was 0.5 µg/mL (prepared in acidified water).

Statistical analysis

Statistical analysis (cluster analysis) was performed using Statistica 12.0 (Statsoft, Poland). The results were presented in the form of dendrograms as well as heat maps.

Results and discussion

LC–MS/MS analysis of honeys

The analysis of free aminoacids in honey samples was based on the LC–MS method which was developed by Özcan and Senyuva (2006) and verified on different food

matrices, such as baby food, fruit juices, milk, nuts, as well as honeys. In modified form, this method was also applied on the analysis of selected amino acids in fine bakery products in order to determine the precursors of acrylamide formation in heated foods (Ciesarová et al. 2009).

In contrast to the Özcan and Senyuva (2006) method, in this study, the positive electrospray ionisation (ESI) was used in order to generate protonated molecular ions, followed by their collision-induced fragmentation to specific product ions. For the measurements in the multiple reaction monitoring mode (MRM), two ion transitions for each amino acid were chosen (one for quantification and the second for confirmation purposes) on the basis of an initial fragmentation study. For each ion transition, the parameters such as fragmentor voltage and collision cell energy were optimized in order to achieve the best chromatographic response. The summary of all transitions as well as optimized MRM conditions is listed in the Table 1.

The linearity of detector response for individual amino acids was checked through calibration curves in the concentration range from 0.02 to 6.00 µg/mL. The calculated coefficients of determination (R²) were above 0.99 for all amino acids. Limits of detection (LOD) and limits of quantification (LOQ) were calculated using the program

Mass Hunter Workstation software (Version B. 04.00). Obtained LOD values ranged from 3.0 ng/mL for valine to 13.0 ng/mL for hydroxyproline and LOQ values from 10.0 ng/mL for valine to 34.0 ng/mL for tryptophan (Table 1).

Basic characteristic of honeys quality

Thirty samples of honey of different botanical origin (acacia, rape, forest, lime and multifloral) were analyzed. Honeys have been obtained from six producers, three from Poland and three from Slovak Republic. For all analyzed honeys water content was below the limit value (20%) (Bogdanov et al. 1999) and was in the range from 14.15 to 19.8%. One sample showed a significant excess of the permissible HMF content. Among the remaining samples the average HMF content was 9.0 mg/kg (from 0.02 to 29.99 mg/kg). Regarding diastase activity it was found that honeys from one producer from Slovakia had very low diastase activity. In case of three samples diastase numbers were much under the limit ($DN \geq 8.0$) (Bogdanov et al. 1999). For other honeys DN was in the range from 9.2 to 40.1. The content of proline is also taken into account when evaluating the qualities of honey. As it was already mentioned, the proline content in honey should be higher than 180 µg/g (Bogdanov et al. 1999) as smaller values may be related to the immaturity of honey or its adulteration. Such low values were found in case of two samples from Poland as well as in one sample from Slovakia. Taking above into account from the final consideration of the amino acid profile of honeys, samples with low qualities were excluded. On the other hand one sample of Slovak lime honey exhibited very high amino acids content and was also excluded from further consideration. Finally, twenty-two honey samples were used to determine the amino acid profile of honeys.

Amino acid profile of Polish and Slovak honeys

Average proline content in the analyzed honeys varied from 140.14 µg/g (rape honey from Slovakia) to 389.66 µg/g for forest honey (honeydew honey) from Poland (Table 2). These are the characteristic values reported for both Polish honeys (Janiszewska et al. 2012) as well as for other European honeys (Kečkeš et al., 2013). In case of Chinese honeys, Chen et al. (2017) discovered higher proline content (an average of about 317 µg/g and a maximum above 600 µg/g) in rape honeys. Interestingly, regardless of geographical origin, contents of proline in rape honeys ranged from 140.14 ± 17.02 to 163.25 ± 10.71 µg/g. According to Szczesna et al. (2011) such low proline content in this type of honey is characteristic for so-called early spring honeys. In the case of

Slovak honey significantly lower content of proline was found in one sample of lime honey (about 166.10 µg/g), also one sample of forest honey was characterized by low proline content (137.91 µg/g). Such adulteration or immaturity can be suspected since forest honeys are usually characterized by high proline content (Bosi and Battaglini 1978). For Polish honeys, in addition to the rape honey, the lowest proline content was found in a single sample of lime (126.36 µg/g) as well as one multifloral (149.82 µg/g) honey. The resulting values may indicate the immaturity of investigated honeys but on the other hand there are reports of a lower values of proline content in some types of honeys and more importantly of diversity of this amino acid content in honeys of the same type. For example for Serbian honeys proline content was in the range from 39 to 632 (acacia honey), 99–617 (lime honey) and from 84 to 450 µg/g for rape honey (Kečkeš et al. 2013). To avoid drawing conclusions based on uncertain samples of honey those samples were excluded from statistical evaluation as it was mentioned above.

In the analyzed set of honeys, besides proline only glutamine was found in similar quantities (from 130.25 to 271.95 µg/g for Polish acacia and forest honeys respectively) other amino acids were presented to a lower extent: glutamic acid (from 10.45 to 36.30 µg/g for Polish acacia and Slovak forest honeys respectively), lysine (from 6.61 to 28.66 µg/g for Polish forest and Slovak acacia honeys respectively), phenylalanine (from 6.26 to 38.94 µg/g for Slovak rape and Polish multifloral honeys respectively), asparagine (from 9.10 to 20.92 µg/g for Polish acacia and Slovak rape honeys respectively), alanine (from 5.48 to 14.19 µg/g for Slovak rape and forest honeys respectively), and valine (from 6.16 to 12.04 µg/g for Slovak rape and forest honeys respectively) (Table 2).

Glutamine content (Table 2) in all analyzed honeys, were significantly higher than the amount reported in the literature for both European (7.61 µg/g for acacia honey to 29.76 for sunflower honey µg/g) (Janiszewska et al. 2012; Kečkeš et al. 2013) and Asian honeys (from 2.21 µg/g for lime honey to 29.95 µg/g for rape honey) (Chen et al. 2017). The relatively high content of this amino acid in all analyzed samples in this experiment compared to results obtained by other authors can be attributed to differences in analytical methods used by the individual authors and certainly requires a comparative study. However, it is worth noting that the glutamine content of approximately 218 µg/g was determined in Spanish honeys by Nozal et al. (Nozal et al. 2004) using gas chromatography with flame ionization detector. Relatively large amounts of this amino acid in Spanish honeys were found also by Paramas et al. (2006) up to 5% of total amino acids pot.

Glutamic acid content determined in acacia and forest honeys was characteristic for these honeys and slightly

Table 2 Average free amino acids content in selected honeys

Country	Poland					Slovakia				
	Acacia	Lime	Rape	Forest	Multifloral	Acacia	Lime	Rape	Forest	Multifloral
Amino acid [$\mu\text{g/g}$]										
Ala	6.25 $\pm$ 1.68	10.84 $\pm$ 3.69	5.78 $\pm$ 2.05	11.62 $\pm$ 3.79	11.49 $\pm$ 5.37	8.85 $\pm$ 4.99	12.96 $\pm$ 0.88	5.48 $\pm$ 0.23	14.19 $\pm$ 6.58	10.56 $\pm$ 0.84
Arg	5.43 $\pm$ 3.65	8.07 $\pm$ 4.89	7.51 $\pm$ 2.46	1.88 $\pm$ 2.02	13.45 $\pm$ 7.44	8.94 $\pm$ 6.61	6.76 $\pm$ 0.33	6.95 $\pm$ 1.64	5.54 $\pm$ 2.31	8.45 $\pm$ 3.70
Asn	5.87 $\pm$ 2.30	3.14 $\pm$ 0.15	3.11 $\pm$ 0.78	4.74 $\pm$ 1.62	5.29 $\pm$ 0.91	8.13 $\pm$ 2.10	9.22 $\pm$ 0.40	3.76 $\pm$ 0.84	6.04 $\pm$ 1.87	4.35 $\pm$ 0.21
Asp	9.10 $\pm$ 2.46	9.47 $\pm$ 4.10	13.47 $\pm$ 3.35	10.22 $\pm$ 4.39	12.75 $\pm$ 0.23	15.83 $\pm$ 5.58	19.92 $\pm$ 1.50	20.92 $\pm$ 1.11	15.30 $\pm$ 9.08	18.60 $\pm$ 0.89
Gln	130.25 $\pm$ 47.94	137.63 $\pm$ 44.24	164.87 $\pm$ 68.27	271.95 $\pm$ 59.45	150.90 $\pm$ 57.15	253.58 $\pm$ 43.15	182.03 $\pm$ 0.52	148.36 $\pm$ 29.21	271.49 $\pm$ 33.44	190.64 $\pm$ 16.02
Glu	10.45 $\pm$ 2.05	15.52 $\pm$ 7.60	11.03 $\pm$ 4.04	24.11 $\pm$ 7.52	21.46 $\pm$ 2.88	25.37 $\pm$ 15.94	22.12 $\pm$ 0.96	13.59 $\pm$ 0.79	36.30 $\pm$ 13.08	29.30 $\pm$ 1.96
Gly	1.73 $\pm$ 0.60	3.02 $\pm$ 1.54	1.52 $\pm$ 0.44	3.40 $\pm$ 0.85	2.85 $\pm$ 1.08	2.42 $\pm$ 1.19	3.12 $\pm$ 0.26	1.28 $\pm$ 0.05	3.49 $\pm$ 1.27	2.47 $\pm$ 0.38
His	4.18 $\pm$ 1.86	3.15 $\pm$ 3.32	4.66 $\pm$ 1.35	0.00 $\pm$ 0.00	3.43 $\pm$ 3.78	6.46 $\pm$ 2.10	6.49 $\pm$ 0.22	4.41 $\pm$ 0.96	2.70 $\pm$ 3.20	5.28 $\pm$ 2.98
Hyp	2.92 $\pm$ 1.83	5.18 $\pm$ 1.46	2.90 $\pm$ 0.28	7.69 $\pm$ 0.68	4.37 $\pm$ 1.63	7.30 $\pm$ 1.40	3.64 $\pm$ 0.16	2.03 $\pm$ 0.55	8.31 $\pm$ 2.66	3.87 $\pm$ 1.13
Ile	4.27 $\pm$ 1.19	6.28 $\pm$ 2.52	5.16 $\pm$ 2.74	4.46 $\pm$ 1.66	11.22 $\pm$ 1.78	3.43 $\pm$ 1.37	4.14 $\pm$ 0.17	3.25 $\pm$ 0.40	5.92 $\pm$ 2.50	7.53 $\pm$ 3.68
Leu	4.64 $\pm$ 1.62	6.87 $\pm$ 3.20	5.50 $\pm$ 2.44	4.95 $\pm$ 1.12	8.68 $\pm$ 0.62	4.58 $\pm$ 2.08	5.47 $\pm$ 0.12	3.65 $\pm$ 0.32	7.40 $\pm$ 3.59	6.71 $\pm$ 0.99
Lys	17.27 $\pm$ 11.23	22.88 $\pm$ 14.05	21.03 $\pm$ 4.20	6.61 $\pm$ 3.95	18.57 $\pm$ 3.52	28.66 $\pm$ 10.20	21.41 $\pm$ 0.81	17.45 $\pm$ 1.99	9.86 $\pm$ 6.30	18.62 $\pm$ 0.23
Met	0.09 $\pm$ 0.09	0.00 $\pm$ 0.00	3.12 $\pm$ 4.41	0.08 $\pm$ 0.12	0.64 $\pm$ 0.39	0.75 $\pm$ 0.90	0.00 $\pm$ 0.00	0.21 $\pm$ 0.24	0.27 $\pm$ 0.32	0.43 $\pm$ 0.02
Orn	0.74 $\pm$ 0.25	0.67 $\pm$ 0.50	0.80 $\pm$ 0.06	0.26 $\pm$ 0.04	0.55 $\pm$ 0.33	1.37 $\pm$ 0.73	1.15 $\pm$ 0.17	0.80 $\pm$ 0.04	0.90 $\pm$ 0.09	0.74 $\pm$ 0.07
Phe	17.11 $\pm$ 10.84	28.71 $\pm$ 10.55	11.33 $\pm$ 4.69	13.15 $\pm$ 4.46	38.94 $\pm$ 24.41	12.97 $\pm$ 7.73	10.78 $\pm$ 0.18	6.26 $\pm$ 0.50	15.22 $\pm$ 5.31	12.50 $\pm$ 4.61
Pro	217.77 $\pm$ 65.90	376.59 $\pm$ 134.28	163.25 $\pm$ 10.71	389.66 $\pm$ 25.92	292.57 $\pm$ 139.83	331.42 $\pm$ 45.24	283.17 $\pm$ 1.84	140.14 $\pm$ 17.02	316.80 $\pm$ 34.12	208.35 $\pm$ 20.79
Ser	3.77 $\pm$ 1.06	6.82 $\pm$ 3.80	3.66 $\pm$ 1.31	5.82 $\pm$ 1.70	5.54 $\pm$ 0.41	5.69 $\pm$ 2.68	7.47 $\pm$ 0.21	3.65 $\pm$ 0.39	8.35 $\pm$ 4.79	7.16 $\pm$ 0.57
Thr	3.96 $\pm$ 1.42	6.28 $\pm$ 3.28	4.31 $\pm$ 1.22	4.73 $\pm$ 0.81	5.77 $\pm$ 0.16	5.33 $\pm$ 1.60	6.44 $\pm$ 0.10	3.41 $\pm$ 0.21	7.76 $\pm$ 3.72	6.34 $\pm$ 0.39
Trp	1.15 $\pm$ 0.68	1.32 $\pm$ 0.52	1.26 $\pm$ 0.57	0.81 $\pm$ 0.04	1.99 $\pm$ 1.27	1.37 $\pm$ 0.68	0.89 $\pm$ 0.05	2.17 $\pm$ 0.70	1.28 $\pm$ 0.43	2.01 $\pm$ 0.49
Val	6.70 $\pm$ 2.97	10.35 $\pm$ 5.32	7.08 $\pm$ 2.05	7.80 $\pm$ 1.23	10.78 $\pm$ 1.28	8.86 $\pm$ 4.05	9.48 $\pm$ 0.06	6.16 $\pm$ 0.65	12.04 $\pm$ 5.29	11.61 $\pm$ 2.18
Total	453.64 $\pm$ 152.36	662.79 $\pm$ 248.76	441.35 $\pm$ 102.70	773.94 $\pm$ 102.09	621.21 $\pm$ 123.63	741.31 $\pm$ 94.65	616.67 $\pm$ 2.98	393.94 $\pm$ 21.26	749.15 $\pm$ 70.46	555.49 $\pm$ 21.07

Fig. 1 Tree diagram of cluster analysis. **a** analysis regarded to geographical origin of honeys, **b** analysis regarded to botanical origin of honeys

Fig. 2 A heat map showing the contribution of individual amino acids in the amino acid profile of honey. **a** analysis regarded to geographical origin of honeys, **b** analysis regarded to botanical origin of honeys

higher compared with data published for Polish (9.12 $\mu\text{g/g}$ for acacia honey and 11.40 $\mu\text{g/g}$ for honeydew honey) (Janiszewska et al. 2012) as well as Chinese acacia honey

(6.84 $\mu\text{g/g}$) (Chen et al. 2017). In our study forest honey from Slovakia contains the glutamic acid at high level of 36.30 $\mu\text{g/g}$ which is in the range observed for Spanish

honeys (from 16.9 µg/g for orange blossom honey to 191.9 µg/g for heather honey) (Nozal et al. 2004).

The total content of free amino acids for Polish honeys (Table 2) ranged from about 441.35 ± 102.70 µg/g (rape honey) to 773.94 ± 102.09 µg/g (forest—honeydew honey), what seems to be characteristic for Polish honeys (390.63 ± 105.34 µg/g for rape honey to 398.41 ± 135.52 µg/g for honeydew honey) (Janiszewska et al. 2012). Total amino acids content for Slovak honeys ranged from 393.94 ± 21.26 µg/g (rape honey) to 749.15 ± 70.46 µg/g (forest honey). It is worth to point out that total amino acids content for Slovak acacia honey was 741.31 ± 94.65 µg/g what was significantly more than in case of Polish acacia honey (453.64 ± 152.36 µg/g).

Cluster analysis of botanical and geographical origin

In order to unify the results prior to performing statistical analysis raw data were subjected to standardization. Standardized data were analyzed by means of agglomeration cluster analysis. This method provided interesting information on the differences and similarities among the analyzed honey. In the first stage of the analysis the geographical origin of honeys has been taken under consideration (Fig. 1a). The shortest bond was estimated between Slovak lime and acacia honeys as well as between Polish rape and acacia honeys (Euclidian bond distance—3.99 and 4.02 respectively). On the similarity of Slovak lime and rape honeys the largest influence had high concentration of aspartic acid and lysine, average and similar concentration of histidine and ornithine as well as low concentration of hypusine and isoleucine. Similarity of Polish rape and acacia honeys were based on low content of alanine, glutamic acid hypusine, proline, serine and threonine as well as valine and average content of histidine lysine and ornithine (Fig. 2a). Another short distance was found between Polish multifloral and lime honey (4.17). Observed relationships were based on high concentrations of phenylalanine as well as valine and alanine, glycine histidine and hypusine in both honeys. The largest distance was observed between Slovak rape honey and both forest honeys from Poland and Slovakia (bond distance 8.6 and 9.5 respectively). Forest honeys were characterized by high concentration of glutamine and hypusine. In contrast for rape honeys those amino acids were in the minority (Fig. 1a and 2a). Although the Polish forest honeys are mainly honeydew honeys they showed similarity to the forest honeys from Slovakia (Euclidian bond distance—5.40) which were mainly nectar honeys.

Close correlation between groups of honeys may suggest phenological similarity (Janiszewska et al. 2012). This observation seems to be obvious in case of rape honeys from both countries (bond distance 4.52, Fig. 1a). The

intensity of flowering and a large area of cultivation this crop, affects the likeness of obtained honey. The results suggest that acacia had a big impact on the amino acid profile of the analyzed multifloral honeys from both countries. It is worth to point out that simultaneously with acacia (*Robinia pseudoacacia*), other melliferous plants bloom, such as common snowberry (*Symphoricarpos albus*) white clover (*Trifolium repens*), large-leaved linden (*Tilia platyphyllos*). So multifloral honeys produced in this period may show some similarity. Especially the period of acacia and lime flowering followed shortly one after another, which could affect the likeness of the amino acid profile of these honeys. Between the period of rape and acacia flowering is a large gap of about 2 weeks so rape should not have influence on this type of honey. However, the possibility of some climatic anomalies should be considered that result in engagement of flowering period of these two plants. In fact, analyzing the similarities of honey without geographical separation (Figs. 1b and 2b) the only cluster was observed in the case of acacia and lime honey. Responsible for this was a similar contribution of such amino acids like arginine, hypusine, lysine as well as glutamic acid in the general amino acid profile of those honeys.

Conclusion

The LC–MS/MS method used for the determination of free amino acids in honeys brings a lot of benefits. It does not require complicated sample preparation including derivatisation steps as well as application of expensive and hazardous chemicals. Performed analysis provide useful information about same similarities between honeys from Poland and Slovak Republic. The analysis of agglomeration showed similarities between different types honeys and especially rape honeys from both countries. Due to high variability among amino acids profile it was impossible to differentiate botanical origin of analyzed honeys just on the basis of amino acid profile, but the analysis of amino acid profile can support the identification of botanical origin of honeys.

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